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(54) Title: COMPOSITION AND METHOD FOR CONTROLLING HEPATITIS C VIRUS INFECTION

(57) Abstract: Disclosed herein are methods and compositions for the treatment and prevention of Hepatitis C Virus (HCV) infection and methods of screening for antiviral agents against HCV infection and/or production. A method of using compositions of certain apolipoprotein-specific monoclonal or polyclonal antibodies to inhibit HCV infectivity is disclosed. Further, methods of using small interfering RNAs (siRNAs) specific to apolipoproteins for treating and/or preventing HCV infection are disclosed. Also disclosed are methods of using siRNAs specific and/or small molecule inhibitors to certain lipoprotein biosynthetic genes and of using recombinant apolipoprotein E and/or their forms of lipoproteins to treat and/or prevent HCV infections. Screening methods for anti-HCV agents include assessing the effect of a candidate agent on apolipoprotein E and/or apolipoprotein C-I gene expression, assembly, and/or secretion and assessing the effect of a candidate agent on the blockage of the interaction and/or incorporation of HCV nonstructural proteins and/or their fusion forms with reporter proteins into HCV virions.

COMPOSITION AND METHOD FOR CONTROLLING HEPATITIS C VIRUS INFECTION

This application claims the benefit of provisional application no. 60/822,354 titled "Composition And Methods For Treating And Preventing Hepatitis C Virus Infection and Screening Methods For Identifying Anti-Hepatitis C Virus Agents" filed Aug. 14, 2006.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

Research for this invention was made with support from the NCI, Grant Number CA093712, and the NIAID, Grant Number AI51592.

FIELD OF THE INVENTION

The present invention relates to the treatment and prevention of Hepatitis C Virus (HCV) infection and screening for antiviral agents against HCV infection and/or production.

BACKGROUND OF THE INVENTION

The hepatitis C virus (HCV) was discovered in 1989 by molecular cloning and has since been recognized as a major cause of viral hepatitis in humans. HCV is a single-stranded positive-sense RNA virus, which is about 9.6 kb in length. HCV belongs to the *Hepacivirus* genus of the family *Flaviviridae*. The viral RNA genome consists of the 5' untranslated region (5'UTR), a single open reading frame (ORF) encoding a viral polypeptide of 3,010-3,040 amino acids, and the 3' untranslated region (3'UTR) of variable length. Upon translation, the viral polyprotein is cleaved by cellular peptidases and viral proteases into core (C), envelope glycoproteins (E1 and E2), P7, non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Sequence analysis and comparison studies have revealed that both the 5'UTR and 3'UTR of the HCV genome are highly conserved. In contrast, sequences of the ORF exhibit a variation among HCV isolates. Based on the nucleotide sequence similarity, HCV has been further grouped into six major genotypes and numerous subtypes.

HCV infection is characterized by the establishment of chronic infection in up to about 85% of individuals exposed to HCV. The chronic HCV infection carries an increased risk of developing fatal liver diseases such as cirrhosis, liver failure, and hepatocellular carcinoma.

HCV-associated end-stage liver disease is the leading cause of liver transplantation in the United States (US). It is estimated that approximately 4 million people in the US and 170 million people worldwide are persistently infected with HCV. Each year, HCV infection results in 8,000-10,000 deaths in the US alone. HCV-related deaths are expected to triple within the next 10-20 years if no effective intervention is made available. Currently, there is no specific and effective therapy to treat HCV infection. Accordingly, there remains an urgent need in the art for specific antiviral targets and agents for effectively treating and preventing HCV infection.

The structure and biochemical compositions of HCV virions have not been determined, although certain studies have found that HCV virions isolated from the plasma of hepatitis C patients were associated with lipoproteins to form lipoviroparticles (LVPs). Apolipoproteins B and E were detected in the low-density fractions of HCV RNA-containing particles, which could also be captured by apolipoprotein-specific antibodies, suggesting an association of the low-density HCV virions with human lipoproteins. However, the roles of apolipoproteins in HCV assembly and production have not been defined.

SUMMARY OF THE INVENTION

The present invention addresses the above identified problems, and others, by providing compositions and methods for treating and/or preventing hepatitis C virus infection in humans. The present invention further includes targets and methods for identification (screening) of effective anti-HCV agents.

The present invention discloses a method of using compositions of apoE- and/or apoC-I-specific monoclonal or polyclonal antibodies to inhibit HCV infectivity. The method further comprises the step of administering an effective amount of the composition to a patient.

The present invention discloses methods of using siRNAs specific to apolipoproteins, for treating and/or preventing HCV infection. The present invention further includes siRNAs specific for certain lipoprotein biosynthetic genes for treating and/or preventing HCV infection.

Also disclosed is a method of using recombinant apoE (E2, E3, and E4) protein and/or their forms of lipoproteins to treat and/or prevent HCV infections.

The present invention further discloses a method of screening for anti-HCV agents by assessing the effect of a candidate agent on apoE and/or apoC-I gene expression, assembly, and/or secretion. The present invention also includes a method of screening for anti-HCV agents by assessing the effect of a candidate agent on the blockage of the interaction and/or incorporation of HCV nonstructural proteins and/or their fusion forms with reporter proteins into HCV virions.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the embodiments, is better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, exemplary illustrations of the invention are shown in the drawings. However, the invention is not limited to the specific methods disclosed herein.

FIGURE 1 is a flow chart illustrating an exemplary method for preventing and/or treating Hepatitis C virus (HCV) infection in accordance with the present invention.

FIGURE 2 is a flow chart illustrating an exemplary method of screening for anti-HCV agents in accordance with the present invention.

FIGURE 3A depicts the results of HCV virion RNA (vRNA) determined by a ribonuclease (RNase) protection assay (RPA) in a sucrose gradient sedimentation analysis of HCV virions in culture media and densities (g/ml) of each fraction.

FIGURE 3B is a Western blot analysis of HCV NS3 protein in cells infected with the different fractions identified in **FIGURE 3A**.

FIGURE 3C depicts the results of RPA used to determine positive-strand HCV RNA in cells infected with the different fractions identified in **FIGURE 3A**.

FIGURE 3D is a Western blot analysis of apolipoproteins B100 (apoB100), C-I (apoC-I), and E (apoE), wherein a density gradient sedimentation analysis of HCV RNA-containing particles is performed as shown in **FIGURE 3A**, and apoB100, apoC-I, and apoE proteins were detected by using apoB-, apoCI- and apoE-specific antibodies.

FIGURE 4A depicts the results of a study carried out to determine the HCV-neutralizing activity of apoE-specific monoclonal antibodies, where HCV positive-strand RNA levels in the Huh7.5 cells infected with HCV in the presence of apoE-specific monoclonal antibodies were determined by RPA.

FIGURE 4B is a graph showing the correlation between HCV-neutralizing activity and concentrations of apoE-specific monoclonal antibodies using the quantitative data derived from **FIGURE 4A**.

FIGURE 4C shows the reduction of infectious HCV titers in the cell culture supernatant of Huh7.5 cells that were infected with HCV in the presence of apoE-specific monoclonal antibodies. The infectious HCV titers were determined by immunofluorescence assay (IFA) as foci-forming units per milliliter (ffu/ml) of cell culture supernatant.

FIGURE 4D depicts the results of an analysis of the inhibition of HCV infectivity in the infectious fractions 3 to 5 of the sedimentation analysis as shown in **FIGURES 3B and 3C** by HCV E2- and ApoE-specific monoclonal antibodies.

FIGURE 5 shows the reduction of HCV infectivity by an apoC-I polyclonal antibody, as determined by Western blot analysis of HCV NS3 protein in cells infected with HCV in the presence of increasing concentrations of apoC-I polyclonal antibody.

FIGURE 6 indicates human apolipoprotein E gene sequences. The protein coding sequences are highlighted in bold letters and 50% gray in color, and the small interfering RNA (siRNA)-targeting sequences are shown in black color.

FIGURE 7A depicts the results of a study conducted to determine the effects of ApoE-specific siRNA and a non-specific control (NSC) siRNA on suppression of HCV virion assembly and production, where the levels of ApoE and apoB proteins in the cell culture supernatants of the HCV-infected and siRNA-transfected cells were determined by Western blot analysis.

FIGURE 7B depicts the correlation between siRNA concentrations and apoE levels in the cell culture supernatants using the quantified data derived from **FIGURE 7A**.

FIGURE 7C depicts the correlation between siRNA concentrations and apoB levels in the cell culture supernatants using the quantified data derived from **FIGURE 7A**.

FIGURE 8A depicts the results of a study conducted to determine the effects of ApoE-specific and NSC siRNAs on suppression of HCV virion assembly and production, where cell culture supernatant was used to infect naïve Huh7.5 cells; At 3 days post-infection, the levels of HCV NS3 protein and positive-strand RNA were determined by Western blot analysis and RPA, respectively.

FIGURE 8B is a graph showing the correlation between siRNA concentrations and HCV positive-strand RNA levels relative to the control (without siRNA transfection) in Huh7.5 cells infected with the supernatants described in **FIGURE 8A**. The data plotted in the graph are derived from the quantification of HCV positive-strand RNA levels in **FIGURE 8A**.

FIGURE 8C depicts the results of infectious HCV titers (ffu/ml) in the supernatants of the HCV-infected and apoE and NSC siRNA-transfected Huh7.5 cells.

FIGURE 9A depicts the results of a study conducted to determine the effects of ApoE-specific and NSC siRNAs on suppression of HCV production, where the virion RNA (vRNA) levels of

HCV RNA-containing particles secreted into the culture supernatants of the HCV-infected and apoE and NSC siRNA-transfected cells were determined by RPA.

FIGURE 9B depicts the results of a study conducted to determine the effects of ApoE-specific and NSC siRNAs on suppression of HCV production, where HCV vRNA levels (%) are plotted against NSC and apoE siRNA concentrations (nM). The vRNA levels relative to control (without siRNA) are calculated as percentage of control (%) using the quantified data derived from **FIGURE 9A**.

FIGURE 10A depicts the results of a study conducted to determine the effects of ApoE-specific and NSC siRNAs on suppression of HCV assembly and production, where intracellular HCV virions were used to infect naïve Huh7.5 cells. At 3 days post-infection, the levels of HCV positive-strand RNA in the infected cells were determined by RPA.

FIGURE 10B depicts the correlation between siRNA concentrations and HCV positive-strand RNA levels relative to the control (without siRNA transfection) in Huh7.5 cells infected with intracellular HCV virions described in **FIGURE 10A**. The data plotted in the graph are derived from the quantification of HCV positive-strand RNA levels in **FIGURE 10A**.

FIGURE 10C depicts the results of infectious HCV titers (ffu/ml) of the intracellular HCV virions isolated from the HCV-infected and NSC and apoE siRNA-transfected Huh7.5 cells.

FIGURE 11 depicts the results of a study conducted to determine the effects of NSC and ApoE-specific siRNAs on HCV RNA replication, where subgenomic HCV RNA-harboring cells were transfected with NSC and apoE siRNAs; and, at 3 days post-transfection, HCV positive-strand RNA levels were determined by RPA.

FIGURE 12 depicts the results of a study to determine the effects of Sterol O-acyltransferase (SOAT or ACAT) and microsomal triglyceride transfer protein (MTP)-specific siRNAs on HCV production, wherein the HCV-infected Huh7.5 cells were transfected with increasing concentrations of NSC, ACAT, and MTP siRNAs, respectively, and, the cell culture

supernatants were used to infect naïve Huh7.5 cells. At 3 days post-infection, HCV RNA was extracted from cells with Trizol reagent, and HCV positive-strand RNA levels were determined by RPA.

FIGURE 13 depicts the structures of CP-346086, glybenclamide, hesperetin, quercetin, and naringenin.

FIGURE 14A depicts the results of a study conducted to determine the effects of CP-346086, a microsomal triglyceride transfer protein (MTP) inhibitor, on suppression of HCV production, where apoB100 and apoE levels in the supernatants of CP-346086-treated cells were determined by Western blot analysis.

FIGURE 14B depicts the results of a study conducted to determine the effects of CP-346086 on suppression of HCV production, where the supernatants of CP-346086-treated and HCV-infected cells were used to infect naïve Huh7.5 cells. At 3 days post-infection, the levels of HCV NS3 protein was determined by Western blot analysis.

FIGURE 14C depicts the results of a study conducted to determine the effects of CP-346086 on suppression of HCV production, where apoB, apoE, and HCV NS3 protein levels derived from quantification of **FIGURES 13A and 13B** are used to plot against CP-346086 concentrations (μM).

FIGURE 14D depicts the results of a study conducted to determine the effects of CP-346086 on suppression of HCV production, where the supernatants of CP-346086-treated and HCV-infected cells were used to infect naïve Huh7.5 cells. At 3 days post-infection, the levels of HCV positive-strand RNA were determined by RPA.

FIGURE 14E depicts the results of a study conducted to determine the effects of CP-346086 on suppression of HCV production, where infectious HCV titers (ffu/ml) in the cell culture supernatants of the CP-346086-treated cells were determined by IFA and are used to plot against CP346086 concentrations.

FIGURE 14F depicts the results of a study conducted to determine the effects of CP-346086 on HCV RNA replication, where subgenomic HCV RNA-harboring cells were treated with CP-346086, and HCV positive-strand RNA levels in the cell were then determined by RPA.

FIGURE 15 depicts the results of an RPA used to determine the effects of glybenclamide, hesperetin, and quercetin on HCV production; where the HCV-infected Huh7.5 cells were treated with increasing concentrations of glybenclamide, hesperetin, and quercetin, respectively; and, the cell culture supernatants were used to infect naïve Huh7.5 cells. At 3 days post-infection, HCV RNA was extracted with Trizol reagent, and HCV positive-strand RNA levels were determined by RPA.

FIGURE 16A depicts the results of a Western blot analysis of HCV NS3 protein in a study to determine the inhibition of HCV infection by a recombinant apoE3 protein.

FIGURE 16B depicts the results of a study to determine the effect of a purified apoC-I protein on the inhibition of HCV infection, where the levels of HCV positive-strand RNA in Huh7.5 cells infected with HCV in the presence of apoC-I protein were determined by RPA.

FIGURE 17A is a Western blot analysis used to assess the inhibition of HCV infection by human high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL).

FIGURE 17B depicts the results of an RPA used to assess the inhibition of HCV infection by human HDL, LDL, and VLDL.

FIGURE 18 is a Western blot analysis of HCV Core (C), NS3, and NS5A proteins, wherein a density gradient sedimentation analysis of HCV RNA-containing particles is performed as in **FIGURE 3A**; The C, NS3, and NS5A were detected by Western blot analysis using C-, NS3-, and NS5A-specific monoclonal antibodies.

FIGURE 19A depicts the construction of infectious HCV expressing an NS5A-GFP fusion protein and fluorescence photographs showing green fluorescence protein (GFP) expression in Huh7.5 cells infected with NS5A-GFP-expressing HCVs. GFP is inserted into NS5A (466 amino acids) at different positions indicated by amino acid numbers (247, 363, and 429).

FIGURE 19B is a Western blot analysis of HCV NS5A or NS5A-GFP fusion protein expression in Huh7.5 cells infected with wild type HCV or mutant HCVs expressing NS5A-GFP fusion protein using NS5A- and GFP-specific monoclonal antibodies.

FIGURES 19C is a Western blot analysis of the NS5A-GFP fusion protein present in HCV RNA-containing particles of the sucrose-gradient fractions using a GFP-specific monoclonal antibody.

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The present invention includes compositions and methods for treating and/or preventing hepatitis C virus infection in humans. The present invention further includes targets and methods for identification (screening) of effective anti-HCV agents.

In accordance with the present invention, HCV assembly and/or HCV infectivity may be controlled by suppressing the levels of certain apolipoproteins (e.g., apoE and apoC-I) in patients, thereby treating and/or preventing hepatitis C virus infection. In accordance with the present invention, HCV may also be treated by targeting certain lipid and lipoprotein biosynthesis genes and/or pathways.

The present invention further includes methods of using monoclonal and/or polyclonal antibodies specific to apolipoprotein E and/or apolipoprotein C-I to treat and/or prevent HCV infection.

The present invention also includes methods of using siRNAs specific to apolipoproteins, including but not limited to apoE, for treating and/or preventing HCV infection. The present

invention further includes siRNAs specific for certain lipoprotein biosynthetic genes, including: acyl coenzyme A:cholesterol acyltransferase (ACAT; also known as sterol O-acyltransferase (SOAT)), and microsomal triglyceride transfer protein (MTP), for treating and/or preventing HCV infection. Such siRNAs may be obtained, for example, from Dharmacon Company (Lafayette, CO).

The present invention also includes a method of using small molecular inhibitors of lipoprotein biosynthesis to treat and/or prevent HCV infections, for example, inhibitors of ACAT and MTP may be used. Examples of ACAT and MTP inhibitors include, but are not limited to: CP-346086, glybenclamide, hesperetin, quercetin, and naringenin.

The present invention additionally includes a method of using recombinant apoE (E2, E3, and E4) protein and/or their forms of lipoproteins to treat and/or prevent HCV infections, including high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL).

The present invention further includes a method of screening for anti-HCV agents by assessing the effect of a candidate agent on apoE and/or apoC-I gene expression, assembly, and/or secretion.

The present invention also includes a method of screening for anti-HCV agents by assessing the effect of a candidate agent on the blockage of the interaction and/or incorporation of HCV nonstructural proteins (e.g., NS5A) and/or their fusion forms with reporter proteins (e.g., Luciferase, alkaline phosphotase, GFP, etc.) into HCV virions.

Turning now to **FIGURE 1**, an exemplary method **100** of the present invention for treating and/or preventing HCV infection in a patient includes the following: providing a composition capable of suppressing apolipoprotein E (apoE) and/or apolipoprotein C-I (apoC-I) levels in the patient **102**; and administering to the patient an effective amount of the composition **104**. An effective amount of the composition to be used in accordance with the present invention is intended to mean a nontoxic but sufficient amount of the composition, such that the desired

prophylactic or therapeutic effect is produced. The exact amount of the composition that is an effective amount will vary from patient to patient, depending, for example, on the age, and general condition of the patient, the severity of the infection being treated, the particular carrier or adjuvant being used and its mode of administration, and the like. Accordingly, the effective amount of any particular composition will vary based on the particular circumstances, and an appropriate effective amount may be determined in each case of application by one of ordinary skill in the art using only routine experimentation.

With continued reference to **FIGURE 1**, the administered composition may include one or more agents capable of suppressing apoE and/or apoC-I. For example, apoE and/or apoC-I may be pre-transcriptionally suppressed **106**, by providing a composition capable of decreasing apoE and/or apoC-I gene expression, such that the relative amount of apoE and/or apoC-I mRNA that is transcribed is decreased **108**. By so reducing apoE and/or apoC-I gene expression, the amount of apoE and/or apoC-I protein that may be produced is decreased, thereby limiting the amount of apoE and/or apoC-I protein that is available to participate in the HCV assembly and production process **110**, resulting in the prevention and/or treatment of an HCV infection in the patient **112**. Examples of agents that may pre-transcriptionally suppress apoE and/or apoC-I include peroxisome proliferator-activated receptors (PPARs), antagonists and interferon.

ApoE and/or apoC-I may also be suppressed post-transcriptionally **114**, for example, by providing a composition capable of degrading the apoE and/or apoC-I mRNA or otherwise blocking translation of apoE and/or apoC-I mRNA, thereby reducing the amount of apoE and/or apoC-I mRNA that is available for translation **116**. By so reducing the amount of mRNA available for translation, the amount of apoE and/or apoC-I protein that may be produced is decreased, thereby limiting the amount of apoE and/or apoC-I protein that is available to participate in the HCV assembly and production process **110**, resulting in the prevention and/or treatment of an HCV infection in the patient **112**. Examples of agents that may post-transcriptionally suppress apoE and/or apoC-I include compounds capable of RNA interference, such as small interfering RNAs (siRNA). RNA interference is a post-transcriptional gene silencing tool, i.e., specifically degrading or destroying the mRNA encoding the product of the gene of interest. Short RNA duplexes that have been shown to successfully interfere with

expression of specific genes of interest in cells are referred to as small interfering RNAs (siRNAs). An siRNA is a double stranded RNA oligonuleotide that is typically less than about 30 nucleotide bases in length. One strand of an siRNA is complementary to a portion of an mRNA of a gene of interest, binds thereto, and degrades mRNAs and/or prevents translation, thereby suppressing expression of the gene.

ApoE and/or apoC-I may additionally be suppressed post-translationally **118**, for example, by providing a composition capable of degrading the apoE and/or apoC-I protein or scavenging the apoE and/or apoC-I protein, thereby limiting the amount of apoE and/or apoC-I protein that is available to participate in the HCV assembly and production process **110**. By so limiting the amount of protein available for participation in the HCV assembly and production process, an HCV infection in the patient may be prevented and/or treated **112**. Examples of agents that may post-translationally suppress apoE and/or apoC-I include acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitors (e.g., glybenclamide and naringenin), Cholesteryl Ester Transfer Protein (CETP) inhibitors (e.g., torcetrapib) and microsomal triglyceride transfer protein (MTP) inhibitors (e.g., CP346086 and BMS-200150). It is also possible that post-translational modification of apoE will affect its incorporation into HCV virions, for instance phosphorylation, and therein results in inhibition of HCV production.

Turning now to **FIGURE 2**, an exemplary screening method **200** of the present invention for identifying anti-HCV agents includes the following: providing cells in culture **202**; uninfecting the cells, infecting the cells with HCV, or HCV-producing cells **204**; treating the cells with a candidate anti-HCV agent **206**; measuring apoE expression in the cells and/or cell culture supernatant or HCV nonstructural proteins (e.g., NS5A) and reporter gene (e.g., luciferase) that is fused with NS5A in the culture supernatant **208**; and identifying the candidate agent as an actual anti-HCV agent when apoE gene expression in the cells and/or supernatant or HCV nonstructural proteins in the culture supernatant is below a predetermined level **210**.

The cells that may be provided **202** include any cells that may be grown in culture, produce apolipoproteins, and/or are capable of being infected with HCV **204**. The cells may be treated with a candidate anti-HCV agent **206** before and/or after infection with HCV, or without

HCV infection, as desired. For example, if an assessment of the abilities of a candidate agent to prevent HCV infection is being made, it may be desirable to treat the cells with the candidate agent before infection with HCV. For another example, if an assessment of the abilities of a candidate agent to treat HCV infection is being made, it may be desirable to treat the cells with the candidate agent after infection with HCV. Additionally, cells without HCV infection can be treated with the candidate agent to assess its effect on apoE expression, assembly, and/or production (secretion).

The apoE and/or apoC-I gene expression level in the cells and/or in the culture supernatant or HCV nonstructural proteins and/or their fusion forms with reporter proteins (e.g., NS5A, NS5A-luciferase, NS5A-GFP, NS5A-alkaline phosphotase, and etc.) in the culture supernatant may be measured **208** by any method known to those skilled in the art. For example, the apoE and/or apoC-I gene expression level could be measured by quantifying the concentration of apoE and/or apoC-I mRNA. For another example, the apoE and/or apoC-I gene expression level could be measured by quantifying the concentration of apoE and/or apoC-I protein in the cells and/or in the cell culture supernatant. For another example, HCV nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) in the cell culture supernatant of the HCV-infected cells could be measured by quantifying the levels of NS (e.g., NS5A) proteins and/or the levels of reporter activity (e.g., luciferase, alkaline phosphotase, GFP, and etc.) of the NS-reporter fusion proteins (e.g., NS5A-luciferase, NS5A-alkaline phosphotase, NS5A-GFP, and etc.) in the culture supernatant.

A candidate agent may be identified as an actual anti-HCV agent when the apoE and/or apoC-I gene expression level and/or HCV nonstructural protein (e.g., NS5A) and the reporter gene that is fused with HCV nonstructural protein (e.g., NS5A-luciferase) is below a predetermined level **210**. The predetermined level may be identified, for example, by quantifying the apoE and/or apoC-I mRNA and/or protein in the cells and/or supernatant using methods known to those skilled in the art and comparing the quantified apoE and/or apoC-I mRNA and/or protein level to a standard curve plotting level of apoE and/or apoC-I mRNA and/or protein. For another example, the predetermined level may be identified using a control sample that does not contain the candidate anti-HCV agent. In this regard, when a control

sample that does not contain the candidate anti-HCV agent is examined concurrently with a test sample that contains the candidate anti-HCV agent, the candidate anti-HCV agent is identified as an actual anti-HCV agent when the detected presence of apoE and/or apoC-I mRNA and/or protein in the test sample is less than the detected presence of apoE and/or apoC-I mRNA and/or protein in the control sample.

The present invention is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of developing and testing the efficacy of the present invention.

EXAMPLES

In the studies described herein, the following is demonstrated: (1) HCV nonstructural proteins NS3 and NS5A or NS5A-reporter (e.g., green fluorescence protein, GFP) fusion protein are incorporated into infectious HCV particles; (2) apolipoproteins (B, C-I, and E) are present in HCV particles and the level of human apolipoprotein E correlate with that of HCV infectivity; (3) antibodies against apolipoprotein E and apolipoprotein C-I efficiently block HCV infection; (4) apoE-, ACAT-, and MTP-specific siRNAs each inhibit HCV production and/or infection; and (5) small molecular inhibitors of the lipid and lipoprotein biosynthesis pathways suppress HCV production.

HCV virions contain human apolipoproteins (e.g., apoB, apoC-I, and and apoE): To determine the properties of HCV RNA-containing particles, a continuous 20-60% sucrose density-gradient sedimentation analysis was performed. HCV RNA-containing particles in each fraction were measured by the level of HCV virion RNA (vRNA) and the HCV infectivity. With reference to **FIGURE 3A**, which depicts the results of a sucrose gradient sedimentation analysis of HCV virions in the culture media, HCV virions were concentrated by ultracentrifugation and then fractionated through 20-60% continuous sucrose density-gradient centrifugation. Fractions (1 ml each) were collected from the bottom to the top. The HCV vRNA in each fraction, numbered (from the top to the bottom) on the top of **FIGURE 3A**, was extracted with Trizol

reagent and determined by an RNase Protection Assay (RPA). RPA is a laboratory test used to identify individual RNA molecules in a sample extracted from cells. The buoyant density (g/ml) of each fraction is indicated at the bottom of **FIGURE 3A**. As shown in **FIGURE 3A**, HCV vRNA was detected in nearly all fractions with peak levels in fractions 4 to 6. The buoyant density of the HCV RNA-containing particles covers a range varying from 1.05 to 1.25 g/ml. With reference to **FIGURE 3B**, Western blot analysis of NS3 was conducted with cells infected by the various fractions. Huh7.5 cells in a multi-well culture plate were infected with 100 µl of each fraction. Cells were lysed at 3 days post infection, and the NS3 protein was detected by Western blot. With reference to **FIGURE 3C**, RPA was used to determine positive-strand HCV RNA in cells infected with the different fractions. Results show that fractions 4, 5, and 6, which contain the highest levels of HCV vRNA, were of highest infectivity. These findings suggest that the lower-density particles on the top of the sucrose gradient were assembled in a form containing components necessary for HCV infectivity. The low density of HCV RNA-containing particles could be due to lipoproteins assembled with HCV virions.

To determine whether HCV RNA-containing particles produced by cell culture in vitro contain human apolipoproteins, Western blot analysis was performed using apoB-, apoC-I-, and apoE-specific antibodies. With reference to **FIGURE 3D**, all apoB, apoC-I, and apoE were detected in the low-density fractions. More significantly, the levels of apoE in various fractions are similar to those of HCV vRNA and infectivity (reference to **FIGURES 3A, 3B, and 3C**). These findings suggest that HCV virions contain human apolipoproteins and that the levels of apoE correlate with the HCV infectivity.

Inhibition of HCV infection by apolipoprotein E-and apoC-I-specific antibodies: As apolipoproteins are assembled in HCV virions, a study was conducted to determine whether apolipoprotein-specific monoclonal antibodies will block HCV infection. The inhibition of HCV infectivity by ApoE-specific monoclonal antibodies, apoC-I polyclonal antibody, and HCV E2-specific monoclonal antibodies was assessed. Huh7.5 cells were infected with HCV in the presence of increasing concentrations (µg/ml) of various apoE-specific monoclonal antibodies (mAb23, A1.4, and mAb30). At 3-hr post-infection (p.i.), HCV and apoE antibodies were removed, and the HCV-infected cells were washed with 1x phosphate buffered saline (1x

PBS) twice. The HCV-infected Huh7.5 cells were then incubated with a cell culture medium, for example, Dulbecco's modified Eagle's medium (DMEM). After 3-day incubation, cell culture supernatants were collected and used for the determination of infectious HCV titer, while total cellular RNAs were extracted with Trizol reagent. With reference to **FIGURE 4A**, the levels of HCV positive-strand RNA were determined by RPA using an $\alpha[^{32}P]$UTP-labeled RNA probe containing a negative-strand HCV 3'UTR that is complimentary to positive-strand HCV RNA. **FIGURES 4A and 4B** show that apoE-specific monoclonal antibodies reduced HCV positive-strand RNA levels in a dose-dependent manner. ApoE monoclonal antibodies also decreased infectious HCV titers by nearly four orders of magnitude (10,000 times) at concentrations up to 50 μg/ml (mAb23 in black bar of **FIGURE 4C**). With reference to **FIGURE 4D**, HCV virions present in different fractions, i.e., fraction 3-fraction 5, as described in **FIGURE 3** above were incubated with antibodies specifically against apoE and HCV E2 during infection. Ten μg/ml of monoclonal antibodies against HCV E2 (CBH-5) and apoE were incubated with the fractions during infection. At 3-hr p.i., cells were washed twice with PBS and incubated with DMEM containing 10% fetal bovine serum. At 3 days p.i., total cellular RNAs were extracted with Trizol reagent. The levels of positive-stranded HCV RNA were determined by RPA. Results show that apoE-specific monoclonal antibody 23 (mAb23) potently blocked the infectivity of HCV virions in all different fractions, reducing HCV infectivity by more than 80% as highlighted by percentage of neutralization at the bottom of **FIGURE 4D**. The sizes of RNA markers are indicated on the left and probes and protected RNA products are highlighted by arrows on the right. ApoE monoclonal antibody inhibited HCV infection at least as potently as HCV E2-specific monoclonal antibody (CBH5). Additionally, apoC-I-specific polyclonal antibody was assessed for its HCV-neutralizing capability. **FIGURE 5** shows that apoC-I antibody remarkably reduced the levels of HCV NS3 protein when present during infection, demonstrating its potent HCV-neutralizing activity. These findings suggest that antibodies against human apolipoproteins can be used as therapeutic and/or prophylactic agents to block HCV infection.

Suppression of HCV infectivity, assembly, and production by small interfering RNAs (siRNAs) against apolipoprotein E as well as lipoprotein biosynthetic genes: Acyl Coenzyme A:Cholesterol Acyltransferase (ACAT, also named Sterol O-acyltransferase-SOAT) and

microsomal triglyceride transfer protein (MTP). To determine whether lipids and lipoproteins are required for HCV assembly and production, small interfering RNAs (siRNAs) were created, which siRNAs are included in certain exemplary compositions made in accordance with the present invention and can be used to practice certain exemplary methods in accordance with the present invention. The siRNAs described in this example are specifically against apoE, ACAT (SOAT), and MTP, which are required for lipoprotein synthesis, assembly and secretion.

With references to **FIGURE 6**, the human apolipoprotein E gene sequences (GenBank accession number: NM_000041) are shown, with the protein-coding sequences in bold and 50% gray in color, and the siRNA targeting sequences in blue.

With reference to **FIGURES 7A-7C,** an apoE-specific siRNA and a non-specific control (NSC) siRNA were studied and apoB and apoE protein levels in the cell culture supernatants of siRNA-transfected cells were determined by Western blot analysis. Huh7.5 cells were infected with HCV and then transfected with siRNAs with apoE-specific or NSC siRNA at concentrations of 0.4, 2, 10, and 50 nM. The levels of apoB and apoE proteins secreted into the culture supernatants (media) were then quantified by Western blot analysis using apoB- and apoE-specific antibodies. **FIGURE 7** shows that ApoE-specific siRNA but not NSC siRNA efficiently reduced apoE expression by more than 90% at concentrations up to 50 nM in a dose-dependent manner. In contrast, apoB expression was unaffected by apoE-specific siRNA or NSC siRNA, demonstrating a specific inhibition of apoE expression by apoE-specific siRNA.

With reference to **FIGURES 8A-8C**, the effects of apoE siRNA on HCV production were studied by determining HCV infectivity of the cell culture supernatant derived from the HCV-infected and siRNA-transfected cells. Huh7.5 cells were infected with HCV and then transfected with apoE-specific siRNA or NSC siRNA. At 3-day post-transfection, the cell culture supernatants were used to infect naïve Huh7.5 cells. At 3-days p.i., the level of HCV NS3 protein and positive-strand RNA were quantified by Western blot analysis and RPA, respectively, as shown in **FIGURE 8A. FIGURES 8A and 8B** show that the levels of HCV NS3 and positive-strand RNA were proportionally lowered by increasing concentrations of apoE-specific siRNA but not NSC siRNA, demonstrating that the knockdown expression of apoE by specific siRNA

efficiently suppressed HCV production. The infectious HCV titers in the culture supernatant were also reduced by apoE-specific siRNA by 100 times at 50 nM concentration as shown in **FIGURE 8C**. To further determine whether the apoE-specific siRNA suppressed HCV assembly and production, HCV vRNA extracted from cell culture supernatants, which reflects the total HCV RNA-containing particles, was quantified by RPA. **FIGURES 9A-9B** show that apoE-specific siRNA but not NSC siRNA remarkably reduced HCV vRNA levels (undetectable at 50 nM by RPA) in the culture supernatants, demonstrating that the knockdown of apoE by specific siRNA efficiently blocked HCV production. Additionally, the intracellular HCV virions were prepared by freezing and thawing cells that were infected with HCV and transfected with siRNA. The infectivity of intracellular HCV virions was measured by the level of HCV positive-strand RNA by RPA in the infected Huh7.5 cells. The infectious titers of intracellular HCV virions were determined by immunofluorescence assay (IFA). **FIGURES 10A-10C** show that apoE-specific siRNA reduced the infectivity of intracellular HCV virions by more than 70% as illustrated in **FIGURES 10A** and **10B** and infectious titer by more than 10-fold as illustrated in **FIGURE 10C** at concentrations up to 50 nM. To rule out the possibility of whether apoE-specific siRNA affects HCV RNA replication, Huh7 cells carrying a subgenomic HCV RNA, which can replicate in the cell but does not produce HCV virions, were transfected with apoE-specific or NSC siRNA. At 3-day post-transfection, HCV RNAs were extracted with Trizol reagent, and the levels of HCV positive-strand RNA were quantified by RPA. As shown in **FIGURE 11,** apoE-specific siRNA did not significantly affect HCV RNA replication, demonstrating that apoE is important for HCV virion assembly but not for RNA replication. Taken together, these findings demonstrate that apoE-specific siRNA efficiently knocked down apoE expression and therein suppressed HCV virion assembly and production.

With reference to **FIGURES 12A** and **12B**, the effects of microsomal triglyceride transfer protein (MTP)- and Sterol O-acyltransferase (SOAT, also named Acyl CoenzymeA:cholesterol Acyltransferase-ACAT)-specific siRNAs on HCV production were studied. HCV-producing cells, were transfected with increasing concentrations (nM) of siRNAs against MTP and ACAT, respectively. At 5 days post-transfection, cell culture supernatant was used to infect naïve Huh7.5 cells. At 3 days p.i., total cellular RNAs were extracted and the levels of positive-stranded HCV RNA were determined by RPA. Results show that MTP- and

ACAT-specific siRNAs reduced HCV production by up to 60% at concentrations up to 50 nM as illustrated in **FIGURE 12B.**

These results are consistent with above-described findings that apolipoproteins incorporated into HCV virions are required for HCV infectivity and virion assembly. Inhibition of apolipoprotein production/assembly and secretion by apoE-, apoC-I-, ACAT, and MTP-specific siRNAs reduced HCV virion production and infection. The studies described in the above example indicate that siRNAs against apolipoproteins and lipoprotein biosynthetic pathways can be used as antiviral agents against HCV infection.

Inhibition of HCV assembly, production, and infectivity by small molecular compounds against lipoprotein production and secretion: In order to determine whether small molecular inhibitors of lipoprotein assembly and secretion can suppress HCV production and infection, several small molecule inhibitors, CP-346086, glybenclamide, hesperetin, and quercetin, of the intracellular lipid and lipoprotein synthesis, assembly, and secretion were examined. The chemical structures of these inhibitors are set forth in **FIGURE 13**. These inhibitors are examples to provide a proof-of-concept that lipid and lipoprotein synthesis, assembly, and secretion are unique targets for antiviral drugs against HCV production and infection. Other drugs include ACAT, MTP, Cholesteryl Ester Transfer Protein (CETP), sterol response element-binding proteins (SREBPs) and SREBP-cleavage activating proteins (SCAP) inhibitors that are currently in preclinical development and/or clinical trials for treating cardiovascular diseases and lowering cholesterol.

With reference to **FIGURES 14A-14F**, studies were carried out to determine the effects of an MTP inhibitor, CP-346086, on lipoprotein production/secretion and HCV production. CP-346086 is a potent MTP inhibitor that efficiently blocks apoB and triglyceride secretion with an IC_{50} of 2.6 nM in HepG2 cells. To determine the effect of CP-346086 on HCV production, Huh7.5 cells were infected with HCV at 37°C for 2 hr and then incubated with varying concentrations of CP-346086 for 4 days. The levels of apoB and apoE in the media were determined by Western blotting. As shown in **FIGURE 14A**, apoB- and apoE-containing lipoproteins displayed different sensitivity to CP-346086 treatment. CP-346086 almost

completely blocked apoB secretion at 1 μM, but did not reduce apoE secretion until above 3 μM. The levels of HCV virions released into the media were initially determined by infectivity assay in cell culture. The naïve Huh7.5 cells were infected with HCV-containing media. At 3 days p.i., the levels of HCV NS3 protein and positive-stranded RNA were quantified by Western blotting and RPA, respectively. Consistent with the levels of apoE reduction by CP-346086, the levels of both HCV NS3 protein and positive-stranded RNA were proportionally reduced by increasing concentrations of CP-346086, resulting in >95% reduction at 50 μM as illustrated in **FIGURES 14B, 14C**, and **14D**. Similarly, CP-346086 lowered the infectious HCV titers by 1, 2, 3, and 4 orders of magnitude (10, 100, 1,000, and 10,000 times) at concentrations of 6.25, 12.5, 25, and 50 μM, respectively, as illustrated in **FIGURE 14E**. To exclude a possible inhibition of HCV RNA replication, CP-346086 was used to treat Huh7.5 cells harboring a subgenomic HCV replicon of genotype 2a, which replicated in the cell but did not produce HCV virions. As shown in **FIGURE 14F**, CP-346086 had no significant effect on HCV RNA replication. These findings demonstrate that inhibitors blocking apoE production and secretion can be used as antiviral agents against HCV infection.

. With reference to **FIGURE 15**, three commercial inhibitors, glybenclamide, hesperetin, and quercetin, of lipoprotein assembly and secretion all significantly decreased the levels of HCV virion production. These inhibitors were used to treat HCV-infected cells. The culture supernatants were then used to infect naïve Huh7.5 cells. At 3 days p.i., the levels of positive-strand HCV RNA were determined by RPA. Results show that all these inhibitors reduced HCV production to certain degrees. These findings demonstrate that inhibitors of lipoprotein assembly and secretion suppressed HCV production, which can be potentially used as antiviral drugs against HCV replication and infection. Our findings also suggest that other inhibitors of ACAT, MTP, CETP, SREBP, and SCAP can be potential anti-HCV drugs.

Inhibition of HCV infection by human HDL, LDL, and VLDL: As described above, HCV virions contain human apolipoproteins. Human apolipoproteins were shown to bind the low-density lipoprotein (LDL) receptor and SR-BI, which serve as receptors/co-receptors for HCV infection. A study was conducted to determine whether recombinant apolipoprotein E and human lipoproteins of various densities would inhibit HCV infection.

With reference to **FIGURES 16A** and **16B**, the effect of recombinant apolipoprotein E3 (apoE3) and purified apoC-I on HCV infection was investigated. Huh7.5 cells were infected with HCV in the presence of increasing concentrations (μg/ml) of apoE3 or apoC-I, as indicated on the top of the figures. At 3-hr p.i., the HCV-infected cells were washed with 1x PBS twice and were incubated with medium for 3 days. With reference to **FIGURE 16A**, the levels of HCV NS3 protein in the cells infected with HCV in the presence of apoE3 were then determined by Western blot analysis using an NS3-specific monoclonal antibody and a cellular beta-actin protein as an internal control. With reference to **FIGURE 16B**, HCV positive-strand RNA in the cells infected with HCV in the presence of apoC-I were quantified by RPA. Results demonstrate that ApoE3 and ApoC-I inhibited HCV infection in a dose-dependent manner.

With reference to **FIGURES 17A** and **17B**, the effect of human HDL, LDL, and VLDL on inhibition of HCV infection was studied. Huh7.5 cells were infected with HCV in the presence of increasing concentrations (μg/ml) of lipoproteins, as indicated on the top of the figures. At 3 hr p.i., HCV/lipoproteins were removed, and cells were washed twice with 1xPBS and incubated with DMEM. With reference to **FIGURE 17A**, at 3 days p.i., cells in one set were lysed for Western blot analysis, and with reference to **FIGURE 17B**, the cells in the other set were lysed for RNA extraction with Trizol reagent and RNA quantification by RPA. The levels of positive-stranded HCV RNA were determined by RPA.

The human HDL, LDL, and VLDL were all found to inhibit HCV infection in a dose-dependent manner when incubated with HCV RNA-containing particles during infection. HDL, LDL, and VLDL are known to contain increasing amounts of apoE. These findings suggest that human lipoproteins containing apoE compete the binding of the HCV receptors/co-receptors with HCV virions.

To determine whether HCV RNA-containing particles produced by cell culture in vitro contain viral nonstructural proteins, Western blot analysis was performed using HCV core-, NS3, and BS5A-specific monoclonal antibodies. With reference to **FIGURE 18**, HCV NS3 and NS5A proteins besides HCV capsid protein Core (C) were detected in HCV RNA-containing particles

in fractions 3-7, consistent with their infectivity, as shown in **FIGURE 3B** and **FIGURE 3C**. These results demonstrate that HCV nonstructural proteins are incorporated into HCV virions, which suggest that HCV nonstructural proteins play important roles in HCV virion assembly and production.

With reference to **FIGURE 19A**, infectious HCVs expressing a fusion protein between NS5A and green fluorescence protein (GFP) were genetically constructed. The coding sequence of GFP was inserted into the NS5A gene, where the insertion site was determined by a random insertion mutagenesis method using a transposon-based EZ-Tn5 In-Frame Linker Insertion Kit from EPICENTRE Biotechnologies. Three sites at amino acid 247, 363, and 429 in NS5A were found to tolerate insertions without ablation of HCV production. The GFP-coding sequence was then inserted into these sites of NS5A gene in the infectious HCV RNA genome. The resulted infectious HCVs were viable and express an NS5A-GFP fusion protein, as determined by the presence of GFP in the HCV-infected Huh7.5 cells, appearing as bright spots in **FIGURE 19A**. The infectious HCV titers, as determined by IFA as foci-forming units per milliliter of cell culture supernatant (ffu/ml) and as indicated at the bottom of **FIGURE 19A**, were lower than wild type HCV by up to 100 times. Wild type HCV are generally around 10^5 ffu/ml. These findings suggest that NS5A is important for HCV assembly and virion production. With reference to **FIGURE 19B**, the NS5A-GFP fusion protein was detected by Western blot using a GFP-specific monoclonal antibody in the HCV-infected cells in comparison to the presence of NS5A in the wild type HCV-infected cells. With reference to **FIGURE 19C**, the NS5A-GFP fusion protein was shown to be efficiently incorporated into infectious HCV particles. Collectively, these findings demonstrate for the first time that HCV nonstructural proteins such as NS3, NS4B, NS5A, and likely NS2 are assembled in the infectious HCV virions, providing targets and means to screen for inhibitors that block the incorporation of HCV nonstructural proteins and their fusion forms with reporter proteins (e.g., NS5A, NS5A-luciferase, NS5A-GFP, NS5A-alkaline phosphotase, etc.) into HCV virions.

A screening method for identifying a candidate agent as an actual anti-Hepatitis C virus agent comprises treating the cells with the candidate anti-Hepatitis C virus agent, and determining whether the levels Hepatitis C virus nonstructural proteins and their fusion proteins

with reporters (reporter activity if it is an enzyme) in a cell culture supernatant is below a predetermined level and/or control. The Hepatitis C virus nonstructural proteins and/or their fusion forms with reporter proteins in the cell culture supernatant is measured by determining the Hepatitis C virus nonstructural protein levels and/or reporter activity when fused with reporter proteins as fusion proteins in the cell culture supernatant.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will also be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and example be considered as exemplary only, and not intended to limit the scope and spirit of the invention.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the Specification and Claims are approximations that can vary depending upon the desired properties sought to be determined by the present invention. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the Examples are reported as precisely as possible. Any numerical value, however, inherently contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

CLAIMS

We claim:

1. A composition for treating and/or preventing Hepatitis C virus infection in a patient, comprising a purified antibody which recognizes apolipoprotein E and/or apolipoprotein C-I, wherein said antibody is one of monoclonal, polyclonal, and humanized recombinant.

2. A method of treating and/or preventing Hepatitis C virus infection in a patient, comprising: providing the composition of claim 1; and administering to the patient an effective amount of the composition.

3. A composition for treating and/or preventing Hepatitis C virus infection in a patient, comprising: an siRNA specific for a apolipoprotein.

4. The composition of claim 3, wherein a target apolipoprotein is selected from a group, including but not restricted to apolipoprotein E.

5. A method of treating and/or preventing Hepatitis C virus infection in a patient, comprising: providing the composition of claim 3; and administering to the patient an effective amount of the composition.

6. A composition for treating and/or preventing Hepatitis C virus infection in a patient, comprising an siRNA specific for a lipoprotein biosynthetic gene, selected from a group, including acyl coenzyme A:cholesterol acyltransferase, Cholesteryl Ester Transfer Protein, and microsomal triglyceride transfer protein.

7. A method of treating and/or preventing Hepatitis C virus infection in a patient, comprising: providing the composition of claim 6; and administering to the patient an effective amount of the composition.

8. A method of treating and/or prevènting Hepatitis C virus infection in a patient, comprising:

providing a composition including a small molecular inhibitor of lipoprotein biosynthesis; and administering to the patient an effective amount of the composition.

9. The method of claim 8, wherein the small molecular inhibitor of lipoprotein biosynthesis is an inhibitor of a lipoprotein biosynthetic gene, selected from a group, comprising acyl coenzyme A:cholesterol acyltransferase, Cholesteryl Ester Transfer Protein, microsomal triglyceride transfer protein, and the gene responsible for apoE lipoprotein synthesis, assembly and/or secretion.

10. The method of claim 8, wherein the small molecular inhibitor of lipoprotein biosynthesis is selected from a group, including CP346086, torcetrapib, glybenclamide, hesperetin, quercetin, and naringenin.

11. A method of treating and/or preventing Hepatitis C virus infection in a patient, comprising: providing a composition capable of suppressing apolipoprotein E levels in the patient; and administering to the patient an effective amount of the composition.

12. The method of claim 11, wherein the composition includes an agent selected from the group including: an agent that is capable of pre-transcriptionally suppressing apolipoprotein E levels; an agent that is capable of post-transcriptionally suppressing apolipoprotein E levels; an agent that is capable of post-translationally suppressing apolipoprotein E levels; and an agent that is capable of post-translationally affecting the modification of apolipoprotein E.

13. A screening method for identifying anti-Hepatitis C virus agents, comprising: providing cells in a culture; treating said cells with a candidate anti-Hepatitis C virus agent; measuring apolipoprotein E expression in the cells and/or supernatant of said cell culture; and identifying the candidate agent as an actual anti-Hepatitis C virus agent when apolipoprotein E gene expression in the cells and/or said supernatant is below a predetermined level.

14. The method of claim 13, wherein the apolipoprotein E gene expression in the cells and/or in the cell culture supernatant is measured by determining the apolipoprotein E mRNA and/or protein.

15. A screening method for identifying anti-Hepatitis C virus agents, comprising: providing cells in culture; treating the cells with a candidate anti-Hepatitis C virus agent; measuring the levels of one or more of Hepatitis C virus nonstructural proteins or reporter activity that is fused with the nonstructural proteins in the cell culture supernatant; and identifying the candidate agent as an actual anti-Hepatitis C virus agent when Hepatitis C virus nonstructural proteins and/or reporter activity, in the case of a fusion protein, if the cell culture supernatant is below a predetermined level and/or control.

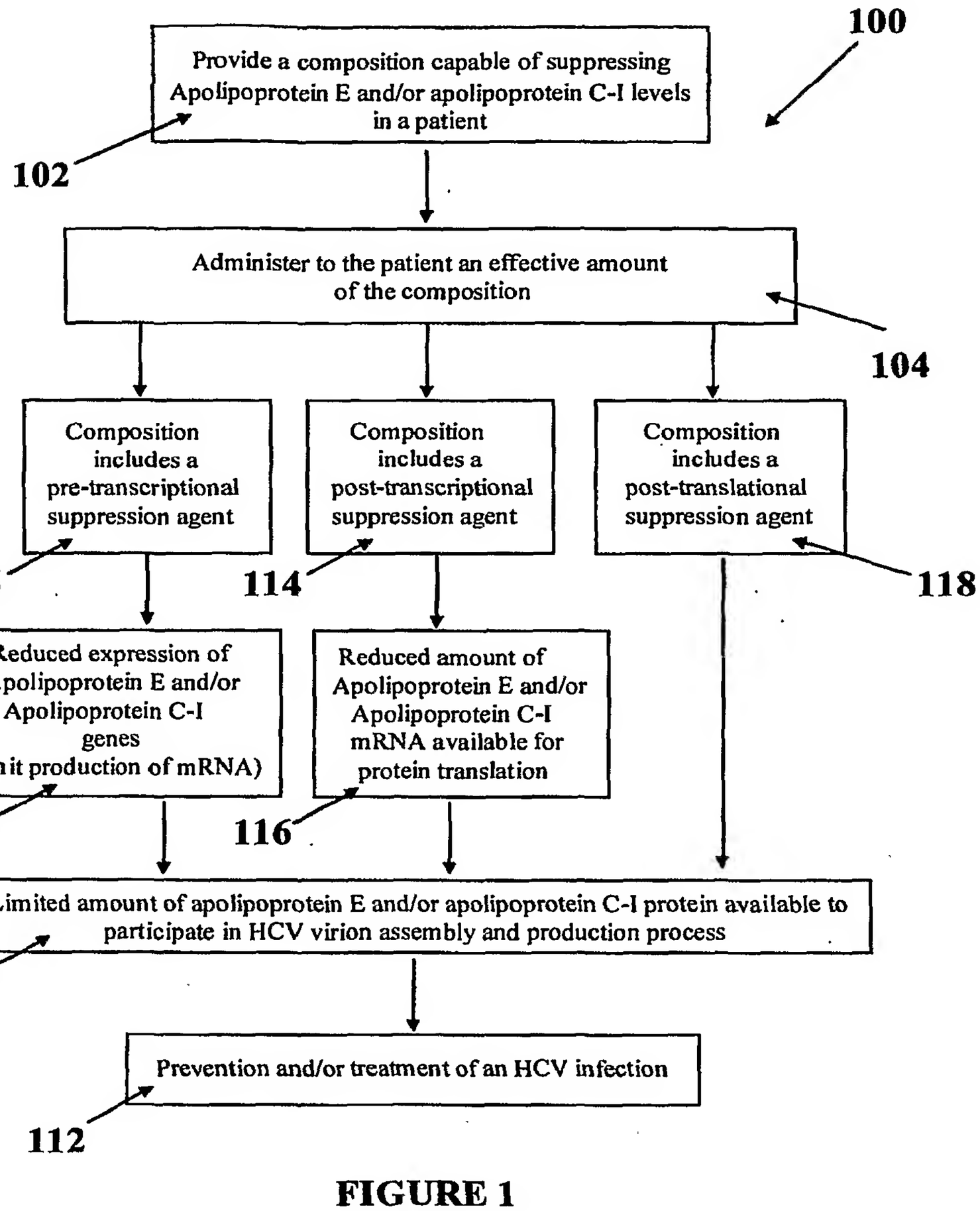
100
Provide a composition capable of suppressing Apolipoprotein E and/or apolipoprotein C-I levels in a patient
102
Administer to the patient an effective amount of the composition
104
Composition includes a pre-transcriptional suppression agent
106
Composition includes a post-transcriptional suppression agent
114
Composition includes a post-translational suppression agent
118
Reduced expression of Apolipoprotein E and/or Apolipoprotein C-I genes (limit production of mRNA)
108
Reduced amount of Apolipoprotein E and/or Apolipoprotein C-I mRNA available for protein translation
116
Limited amount of apolipoprotein E and/or apolipoprotein C-I protein available to participate in HCV virion assembly and production process
110
Prevention and/or treatment of an HCV infection
112

FIGURE 1

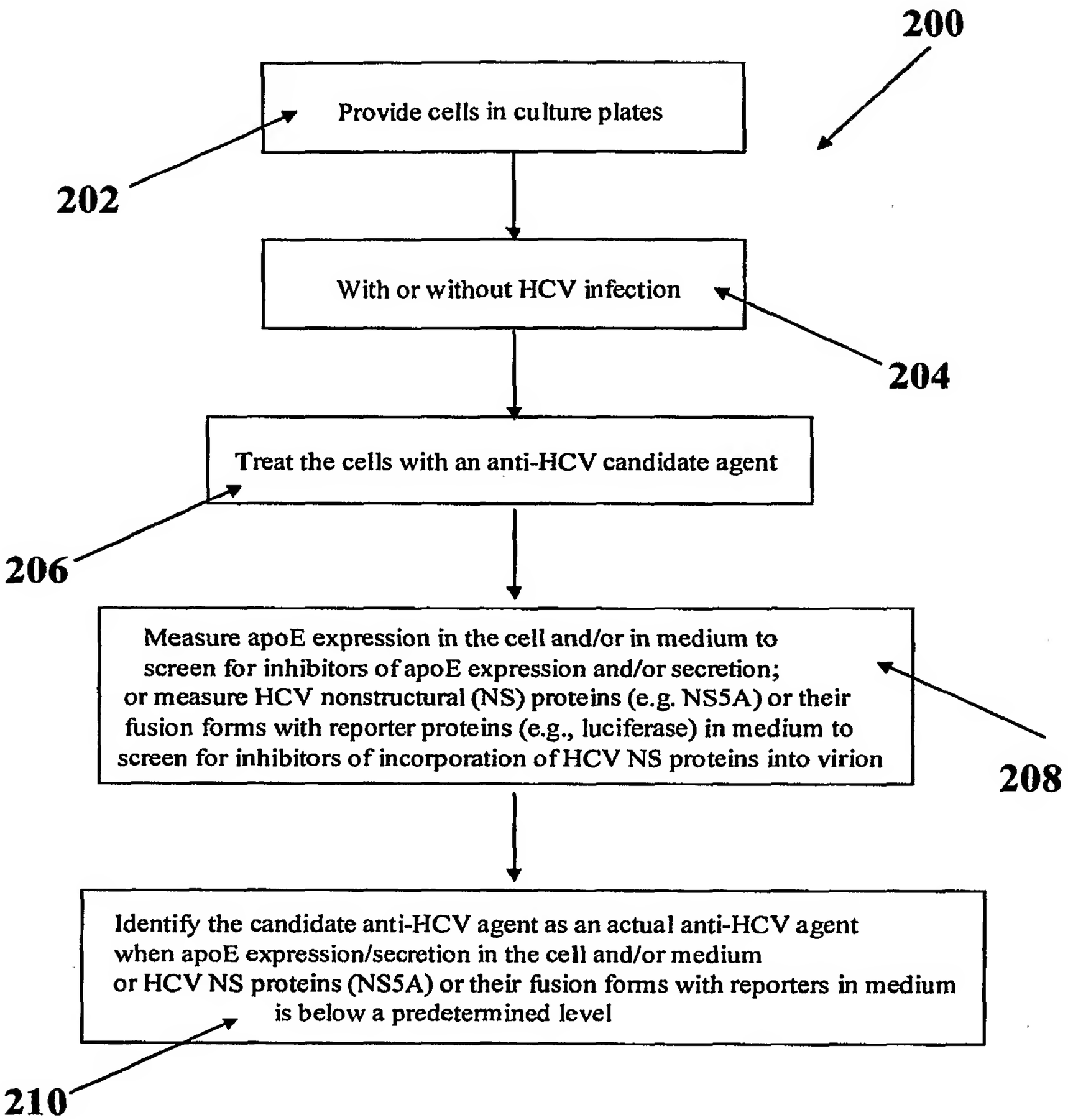
200
Provide cells in culture plates
202
With or without HCV infection
204
Treat the cells with an anti-HCV candidate agent
206
Measure apoE expression in the cell and/or in medium to screen for inhibitors of apoE expression and/or secretion;
or measure HCV nonstructural (NS) proteins (e.g. NS5A) or their fusion forms with reporter proteins (e.g., luciferase) in medium to screen for inhibitors of incorporation of HCV NS proteins into virion
208
Identify the candidate anti-HCV agent as an actual anti-HCV agent when apoE expression/secretion in the cell and/or medium
or HCV NS proteins (NS5A) or their fusion forms with reporters in medium is below a predetermined level
210

FIGURE 2

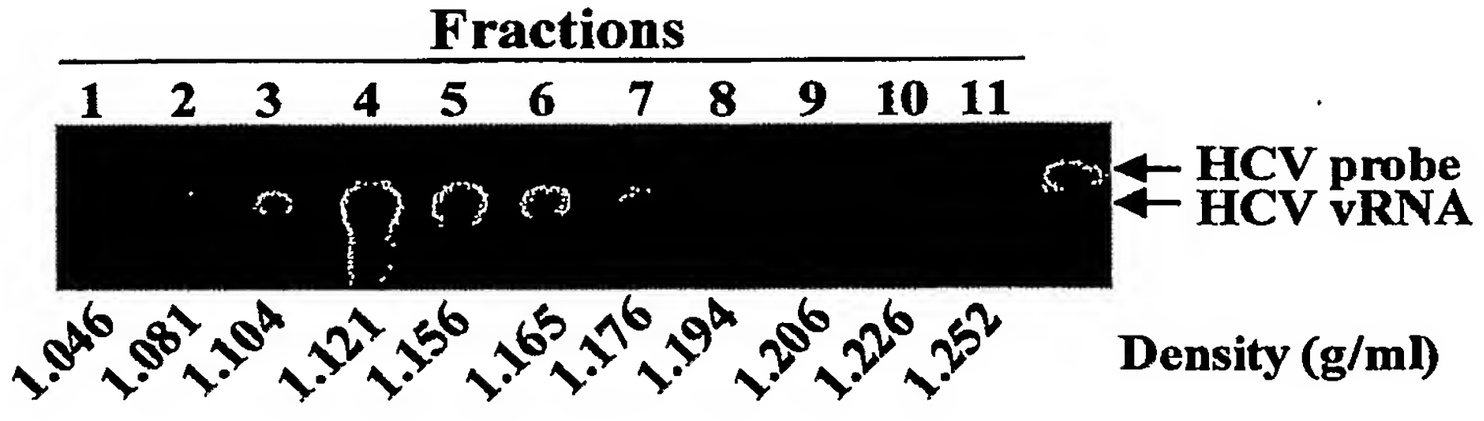

FIGURE 3A

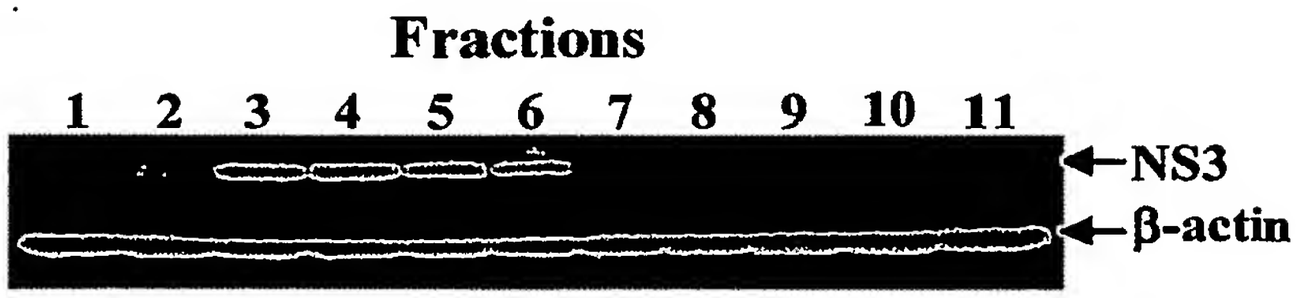

FIGURE 3B

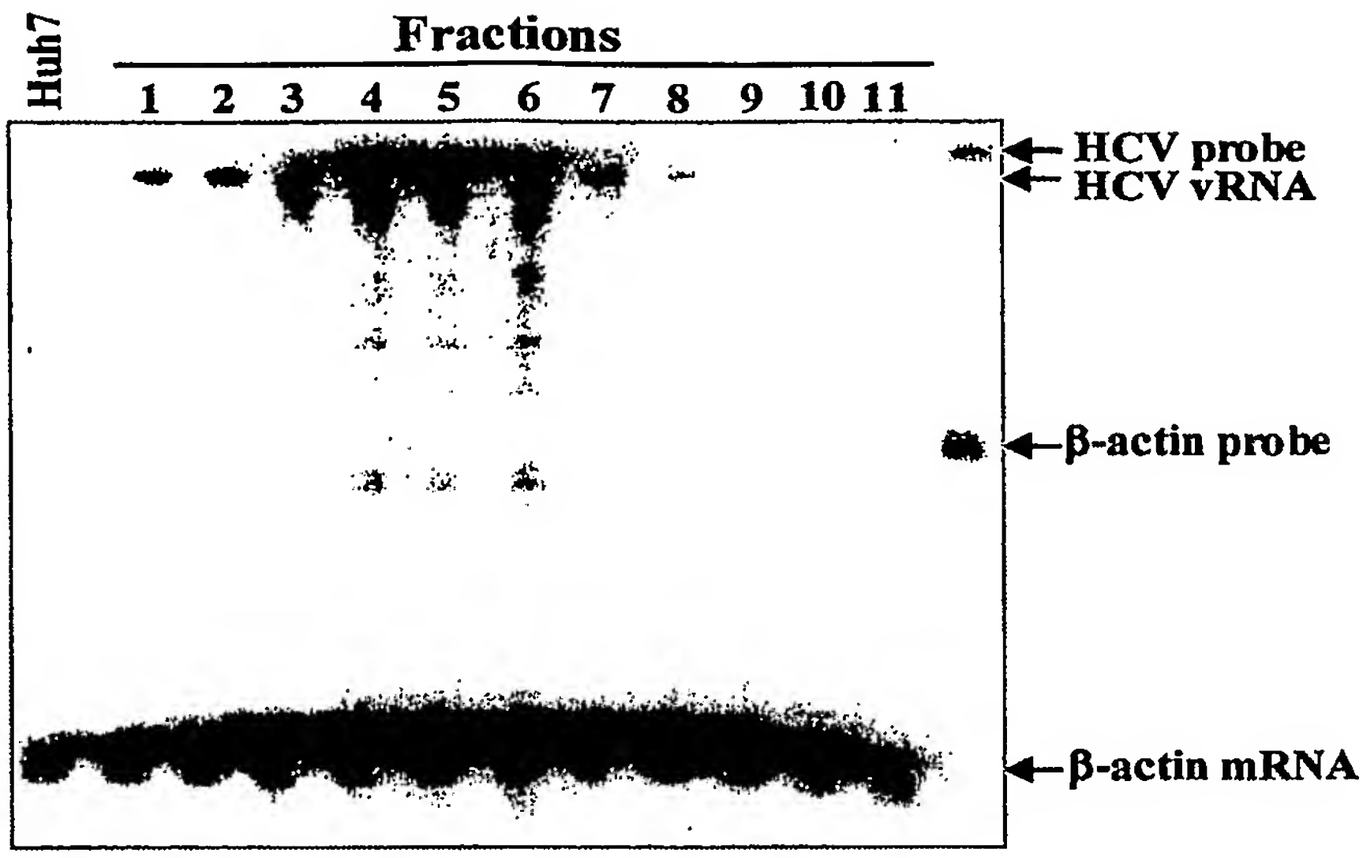

FIGURE 3C

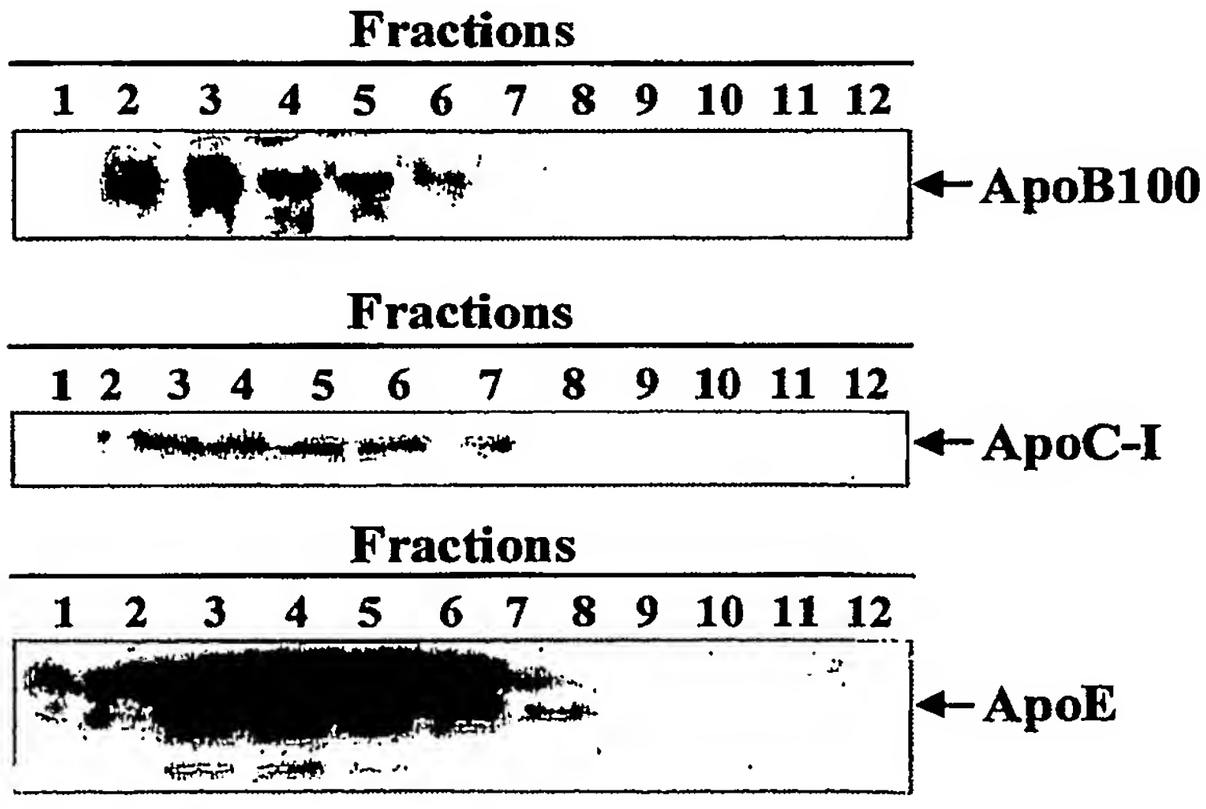

FIGURE 3D

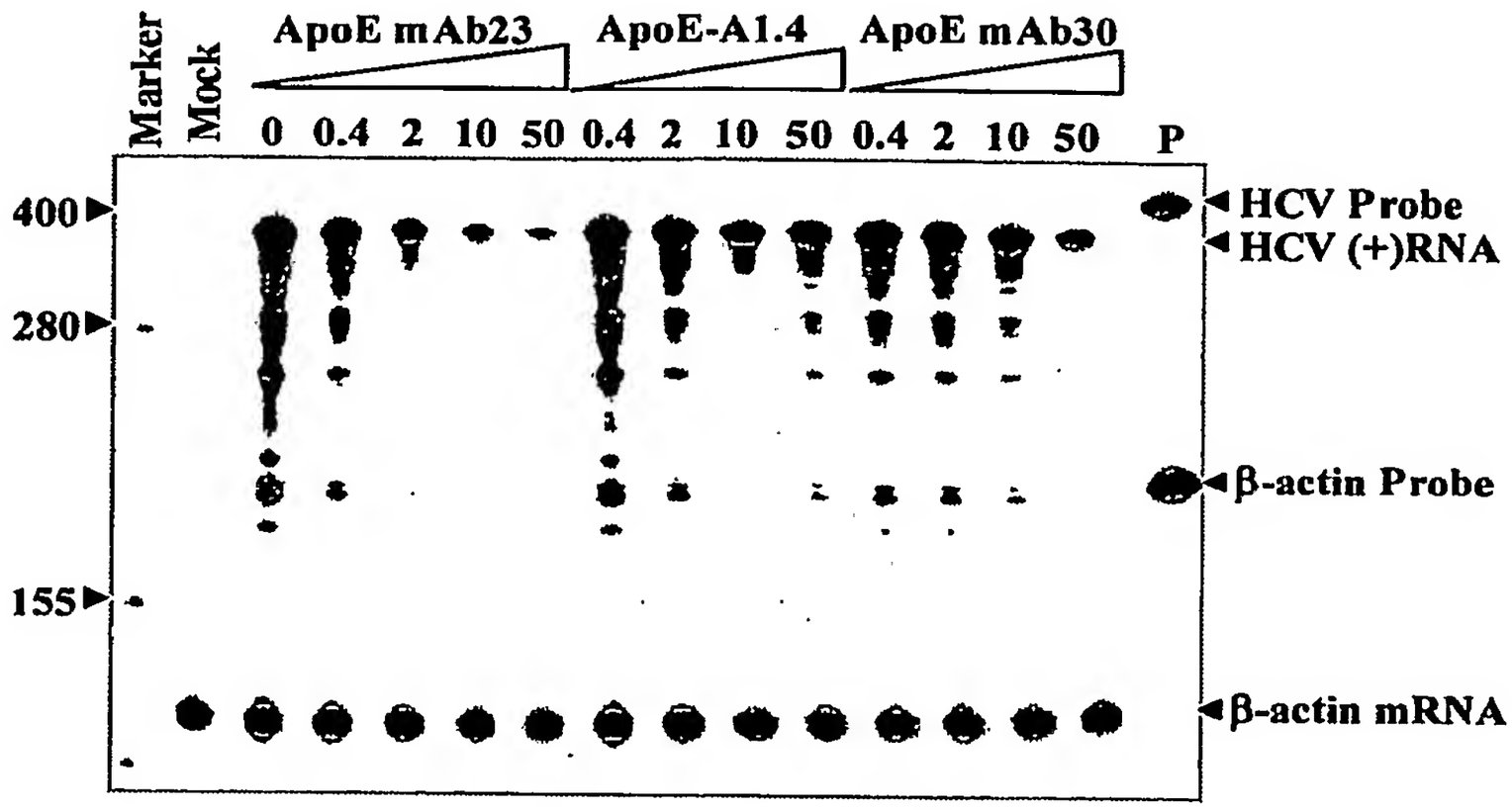

FIGURE 4A

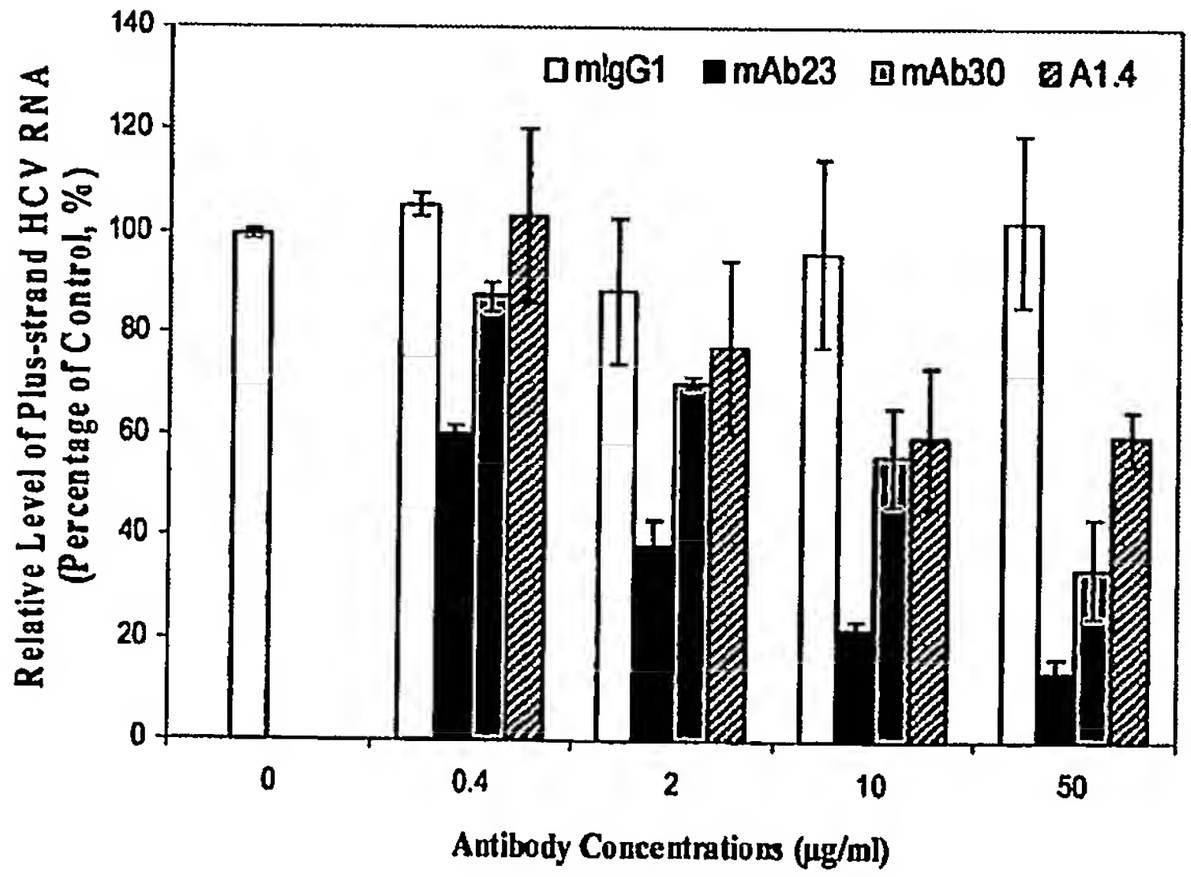

FIGURE 4B

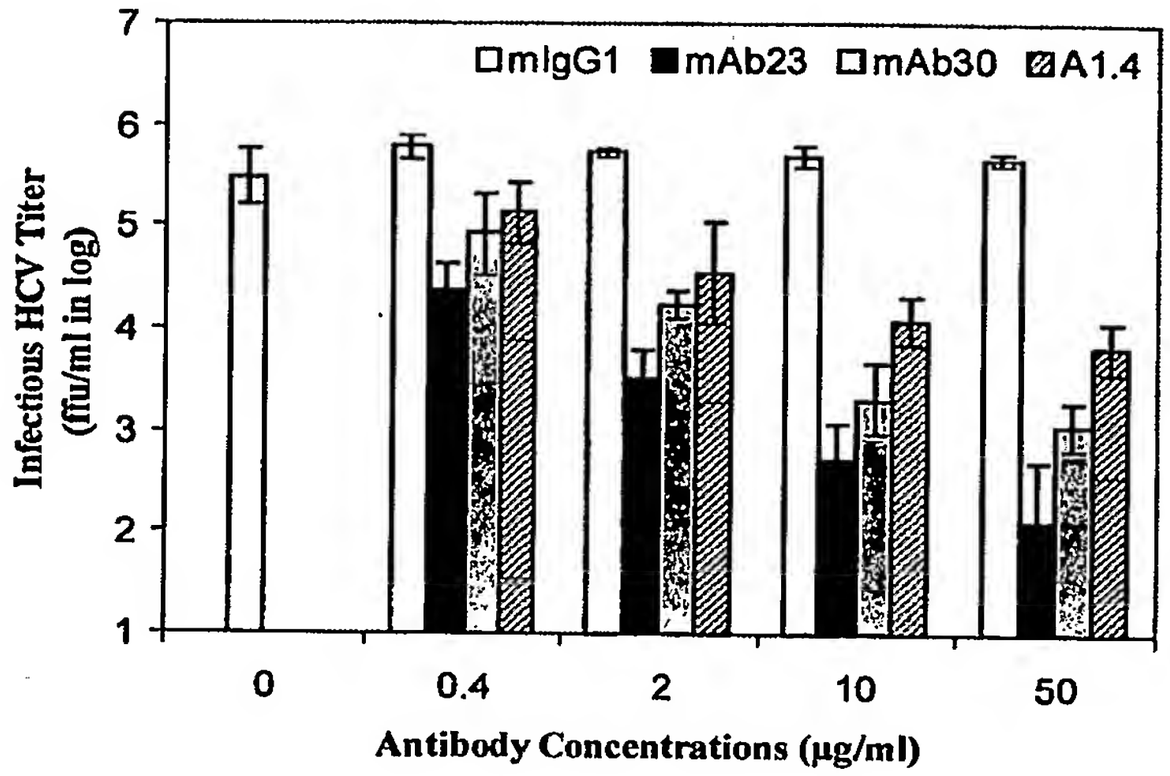

FIGURE 4C

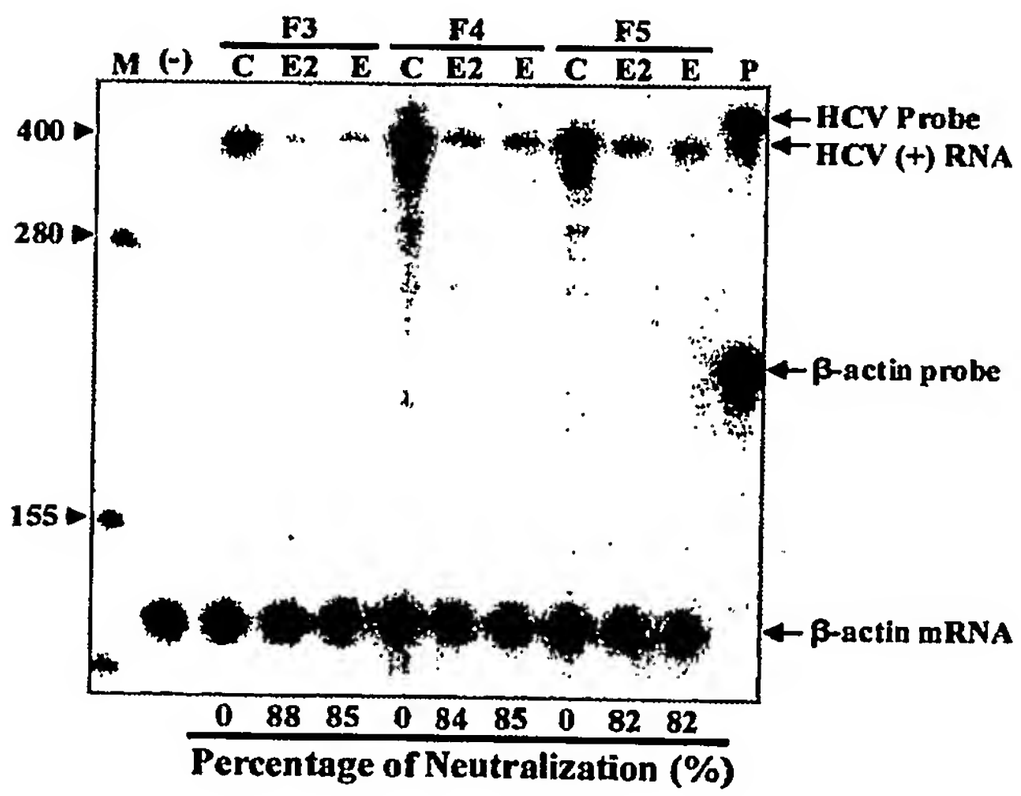

FIGURE 4D

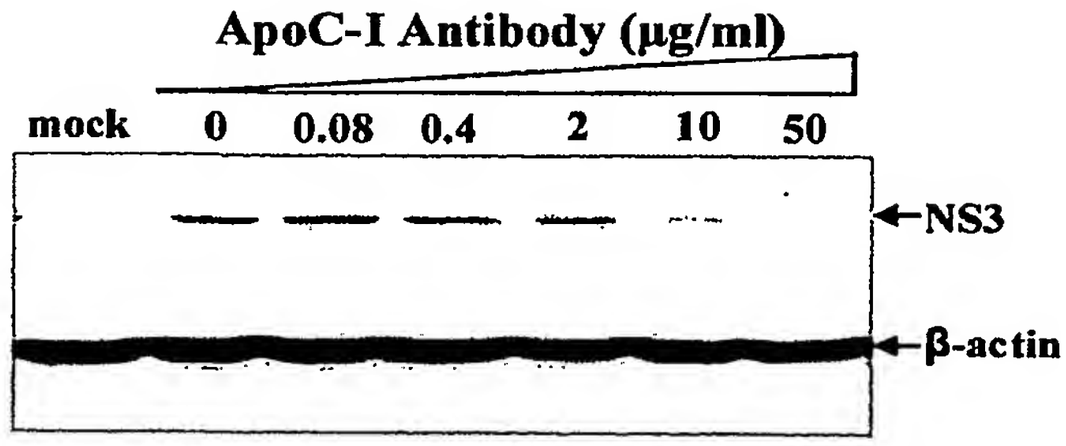

FIGURE 5

Human apolipoprotein E sequence (GenBank Accession number: NM_000041)

```
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 121 tggcaggatg ccaggccaag gtggagcaag cggtggagac agagccggag cccgagctgc
 181 gccagcagac cgagtggcag agcggccagc gctgggaact ggcactgggt cgcttttggg
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 361 aatcggaact ggaggaacaa ctgaccccgg tggcggagga gacgcgggca cggctgtcca
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 661 gcgccatccg cgagcgcctg gggcccctgg tggaacaggg ccgcgtgcgg gccgccactg
 721 tgggctccct ggccggccag ccgctacagg agcgggccca ggcctggggc gagcggctgc
 781 gcgcgcggat ggaggagatg ggcagccgga cccgcgaccg cctggacgag gtgaaggagc
 841 aggtggcgga ggtgcgcgcc aagctggagg agcaggccca gcagatacgc ctgcaggccg
 901 aggccttcca ggcccgcctc aagagctggt tcgagccccc ggtggaagac atgcagcgcc
 961 agtgggccgg gctggtggag aaggtgcagg ctgccgtggg caccagcgcc gcccctgtgc
1021 ccagcgacaa tcactgaacg ccgaagcctg cagccatgcg accccacgcc accccgtgcc
1081 tcctgcctcc gcgcagcctg cagcgggaga ccctgtcccc gccccagccg tcctcctggg
1141 gtggacccta gtttaataaa gattcaccaa gtttcacgca aaaaaaaaaa aaaaaaaaaa
1201 aaaaaaaaaa aaaaaaaaaa aaa
```

Letters with bold faces are apoE-coding sequences, and the siRNA-targeting region is shown as blue.

FIGURE 6

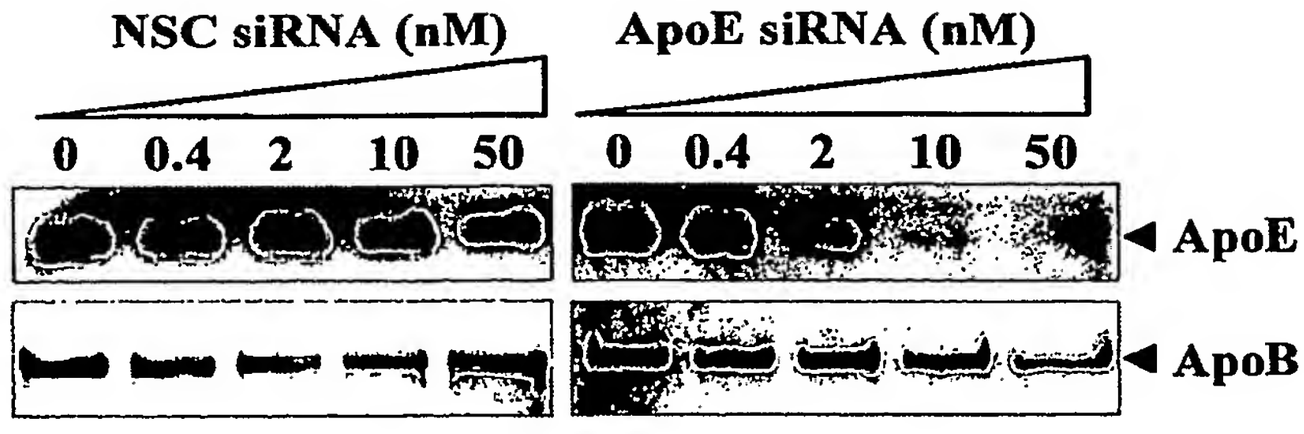


FIGURE 7A

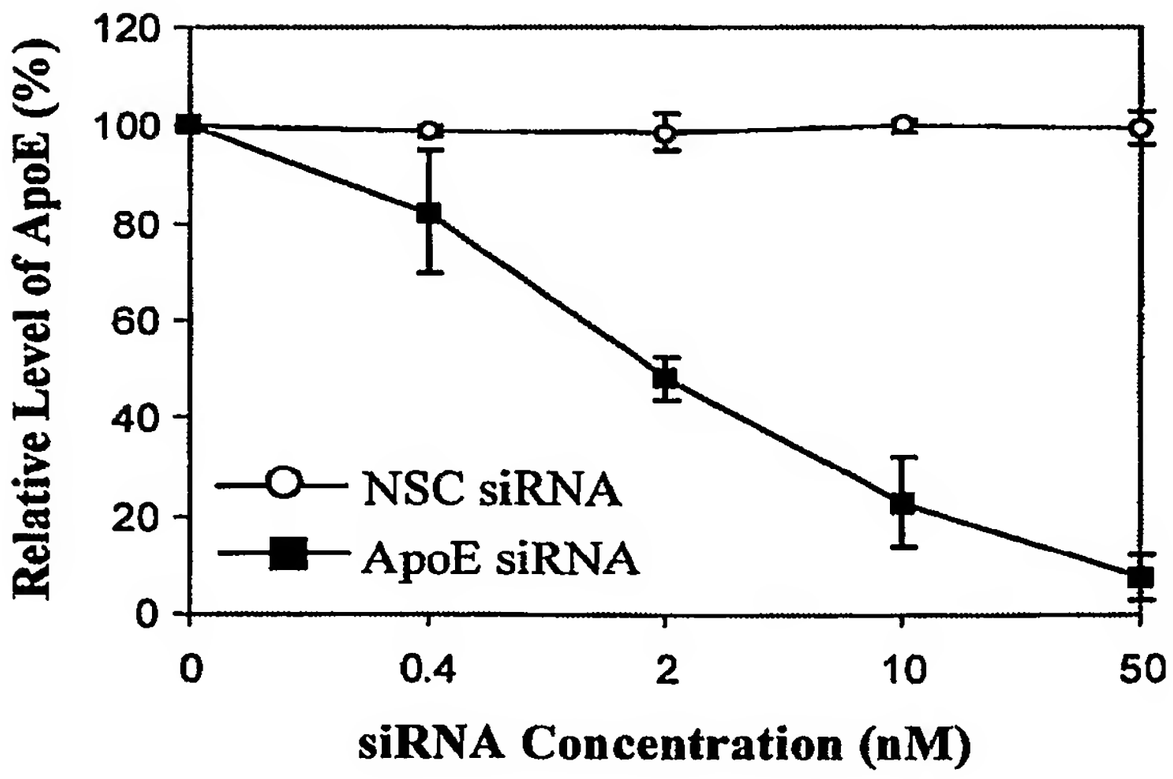


FIGURE 7B

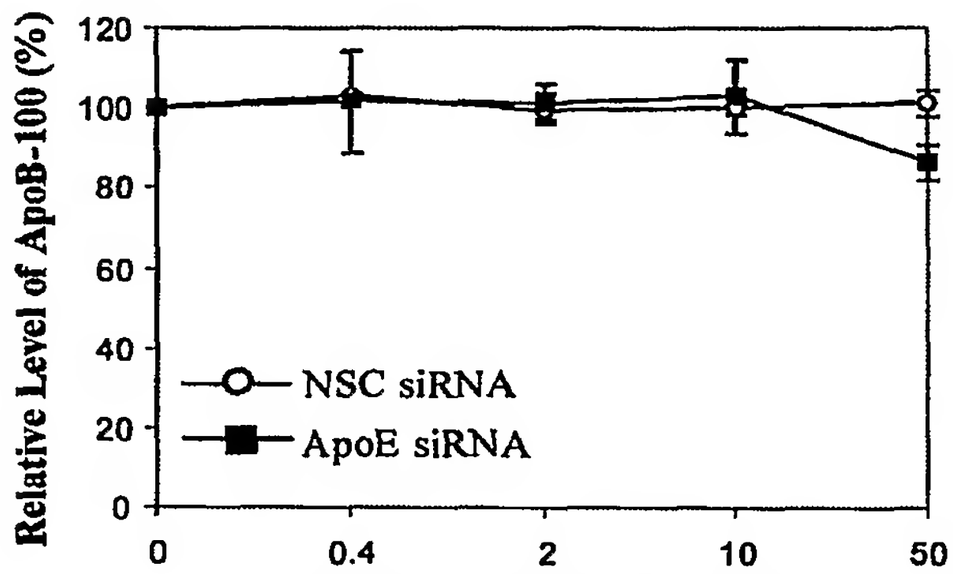


FIGURE 7C

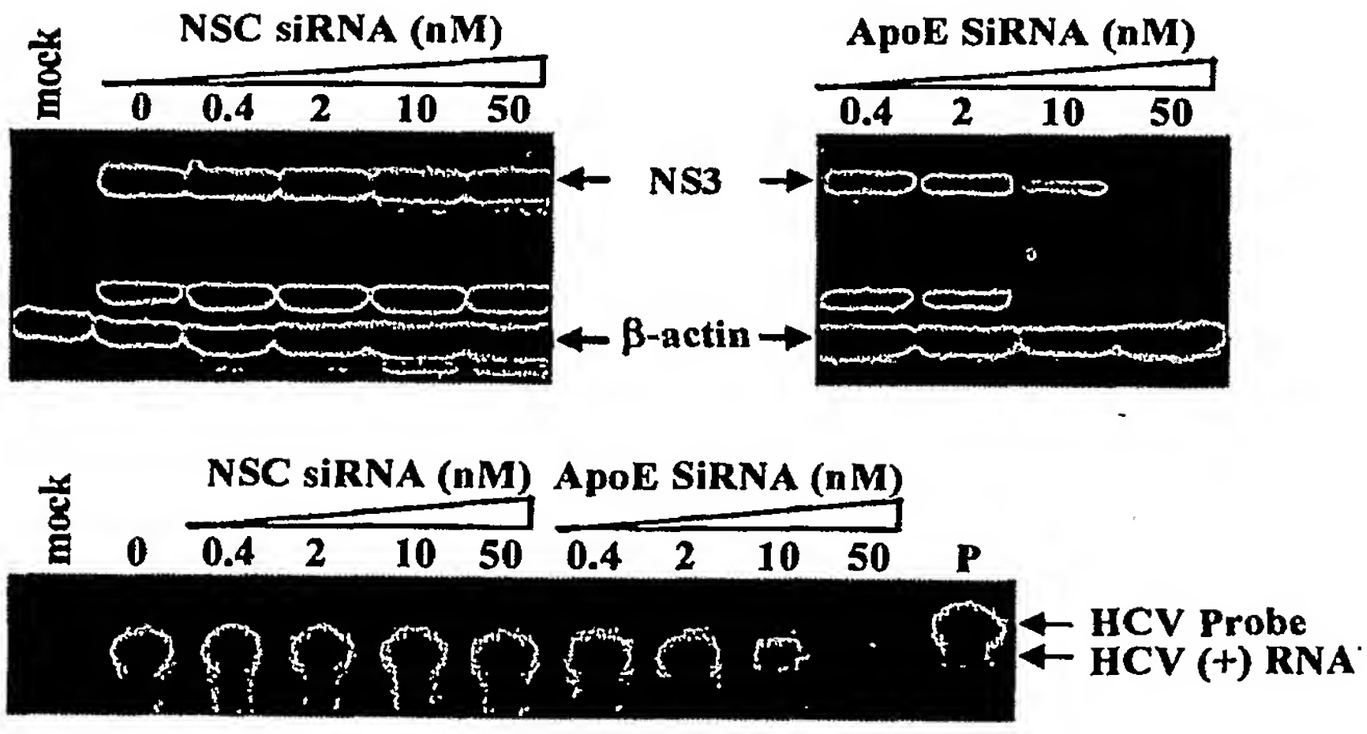


FIGURE 8A

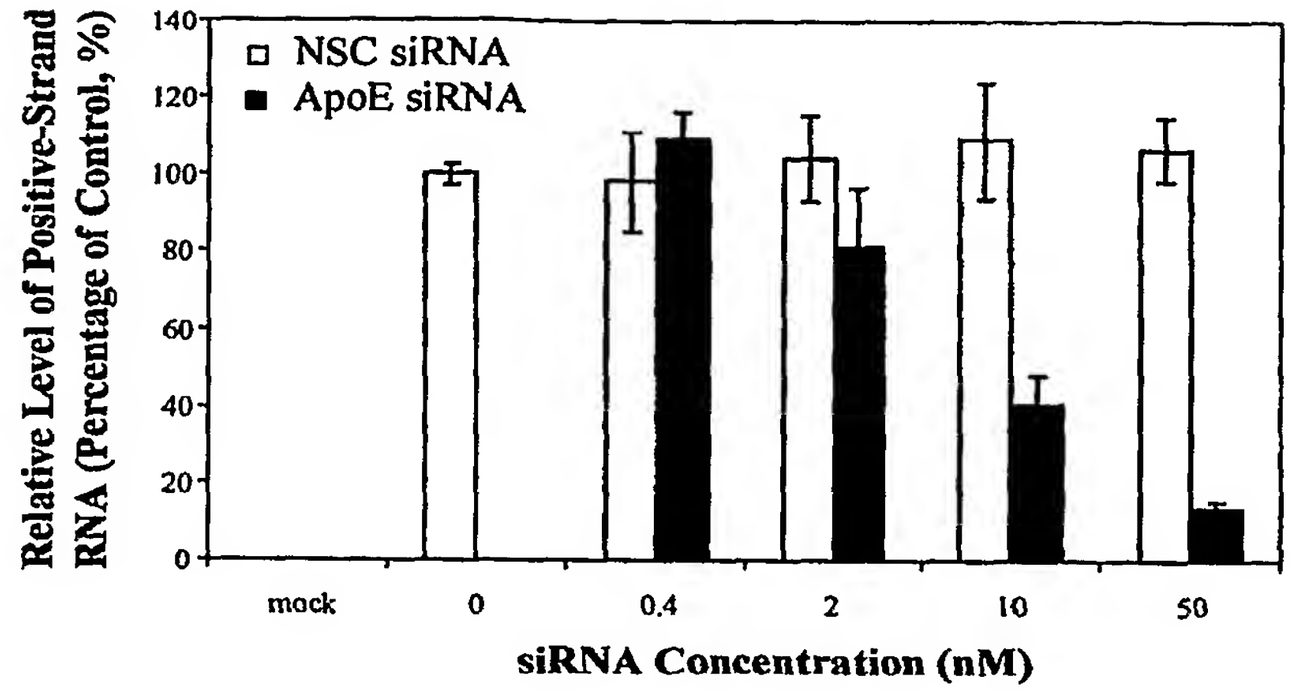


FIGURE 8B

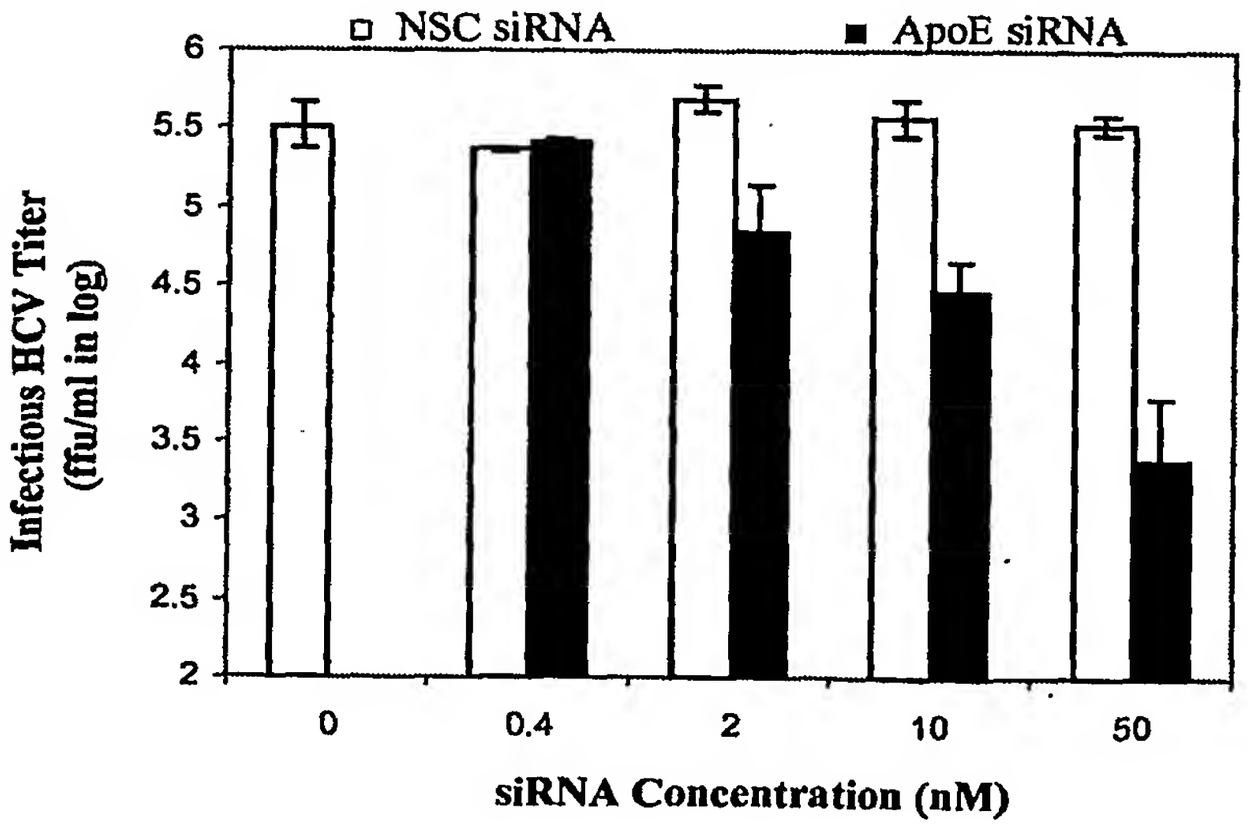


FIGURE 8C

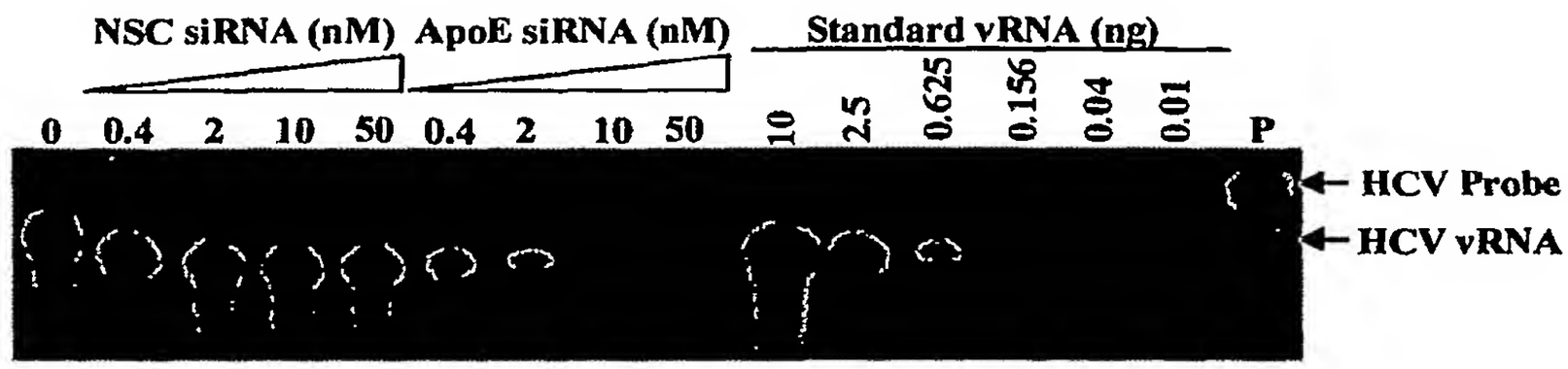


FIGURE 9A

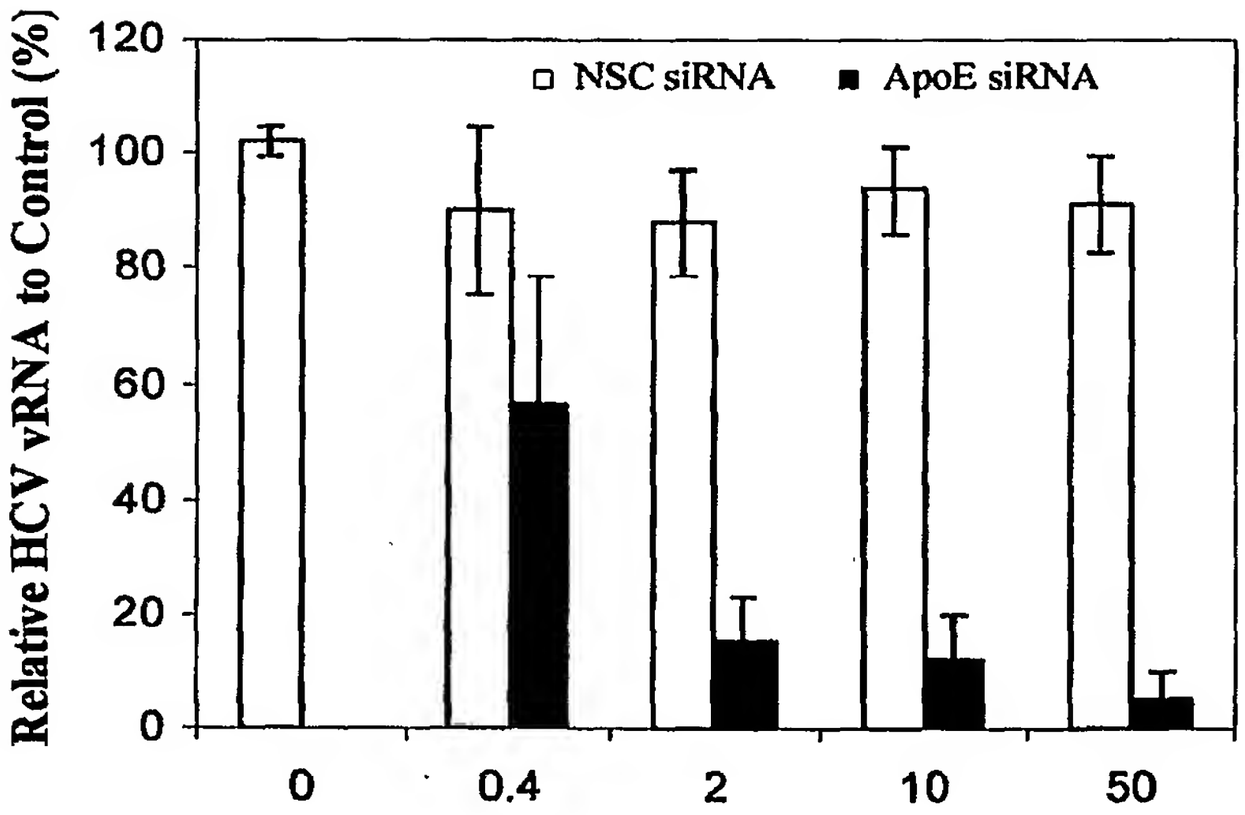


FIGURE 9B

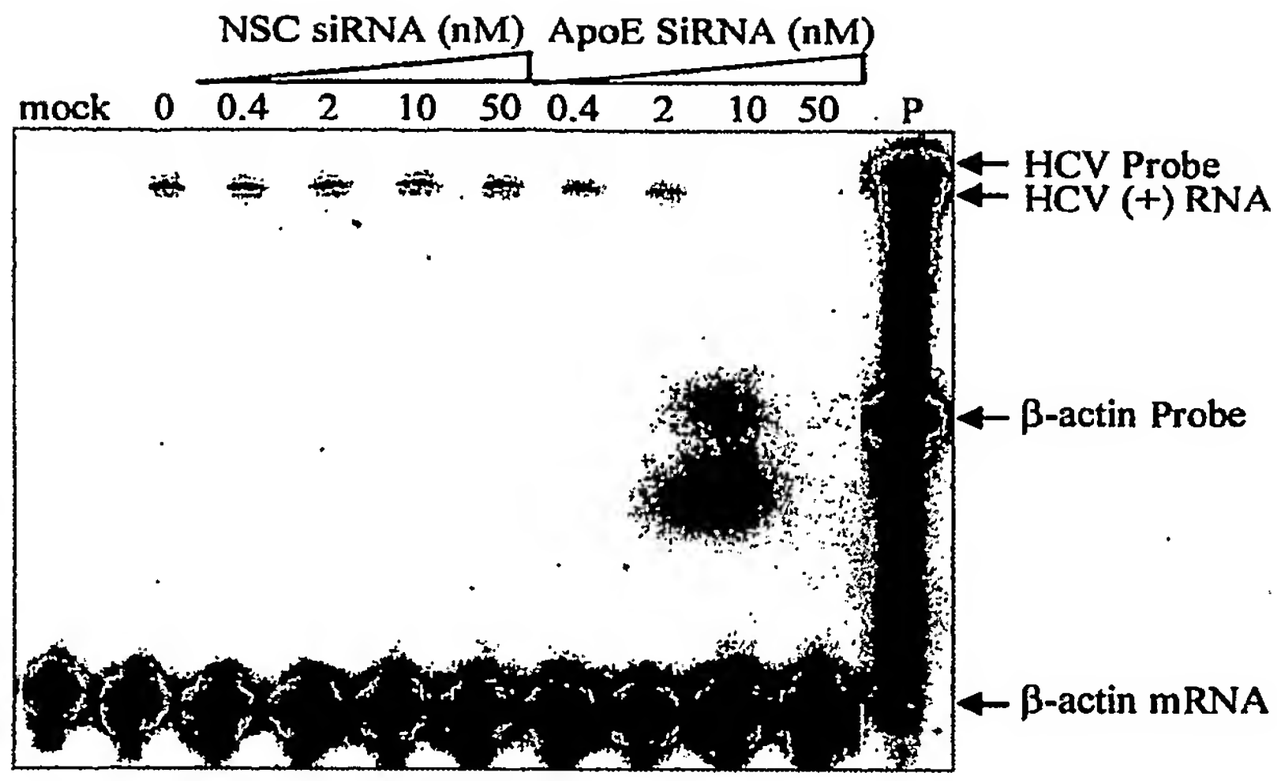


FIGURE 10A

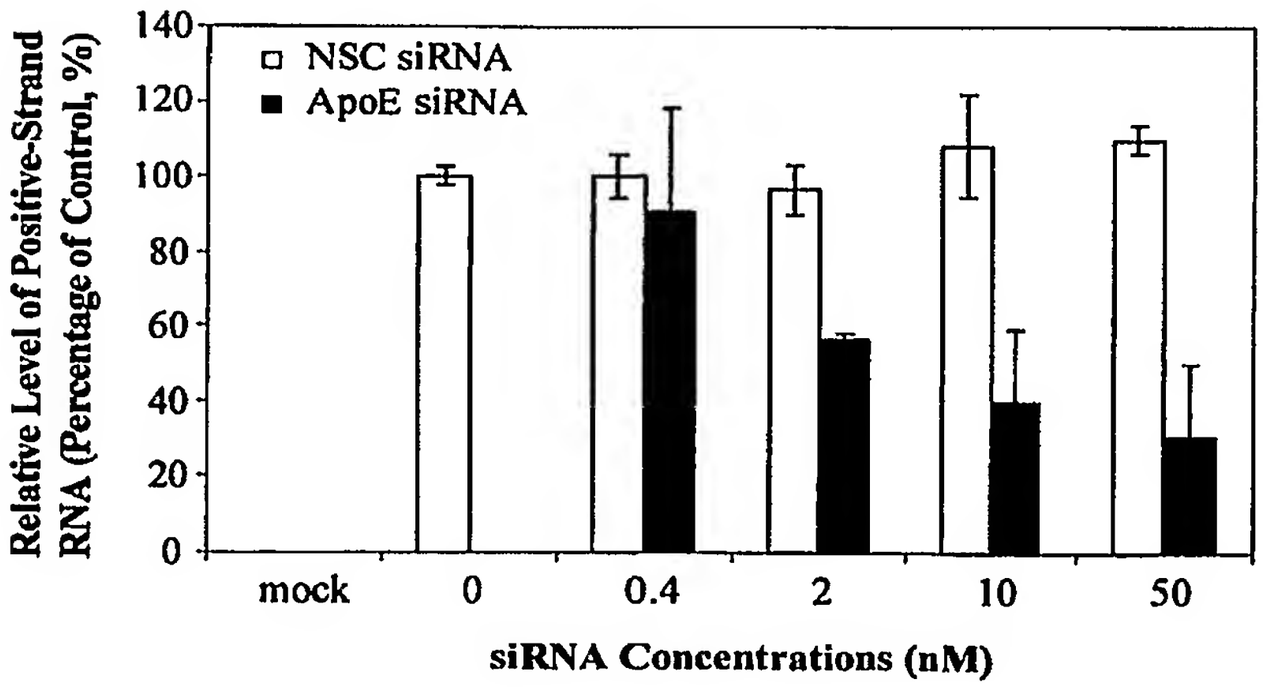


FIGURE 10B

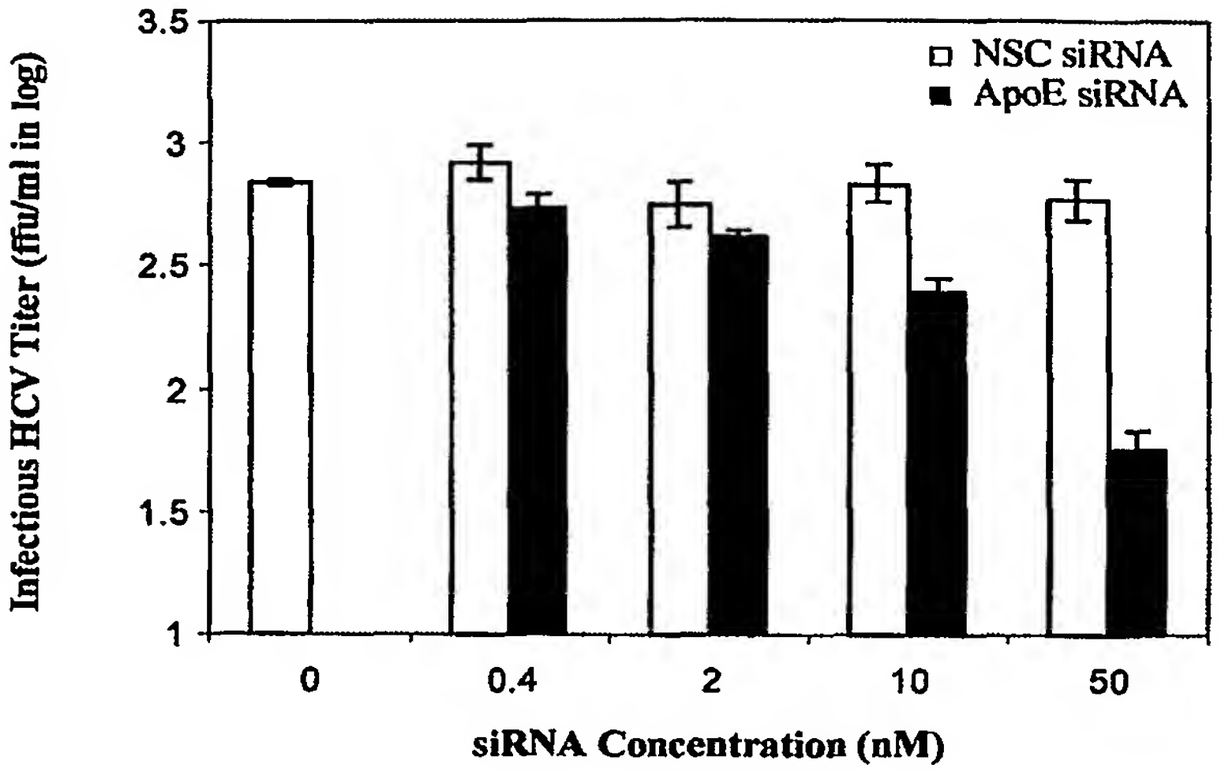


FIGURE 10C

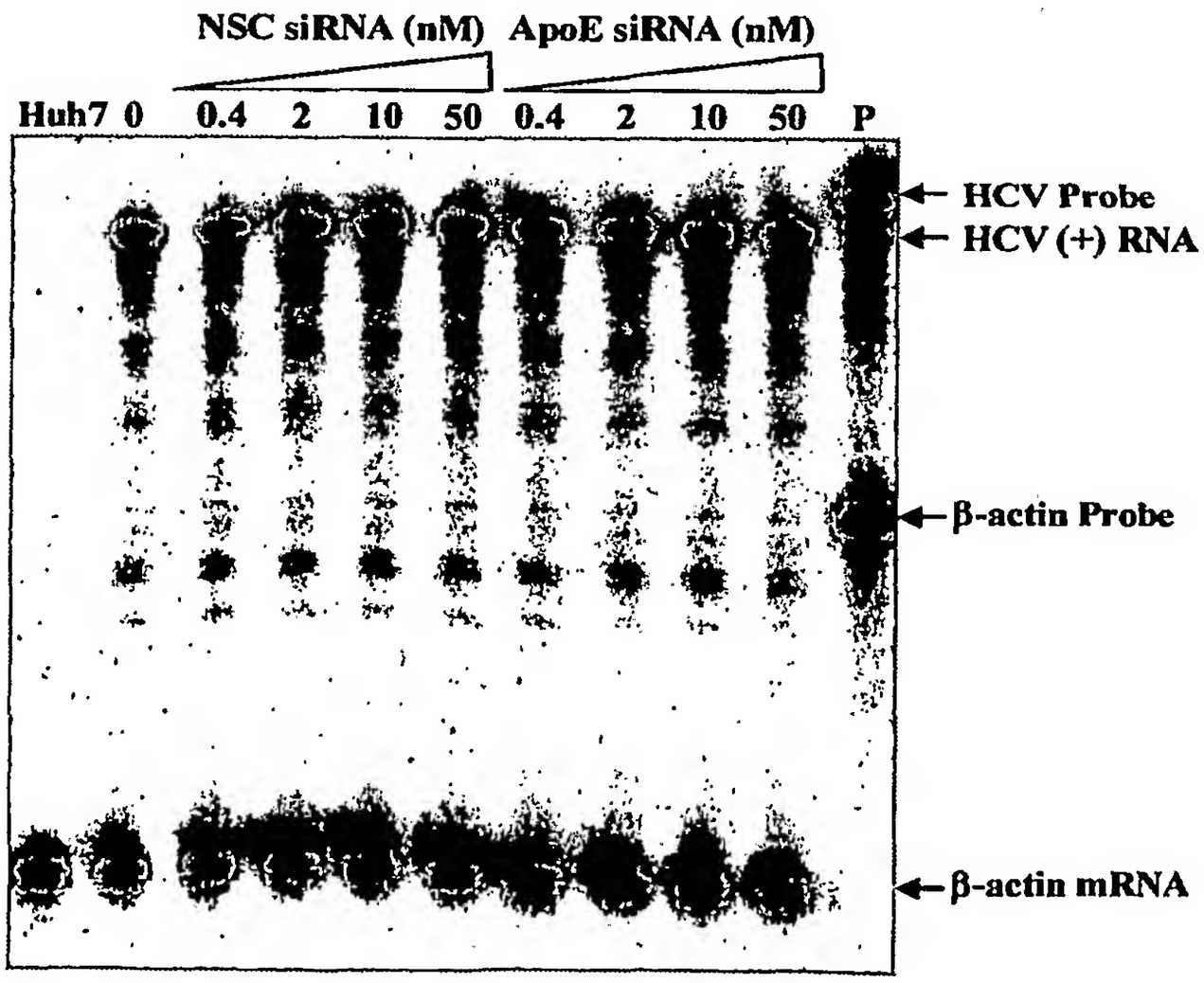


FIGURE 11

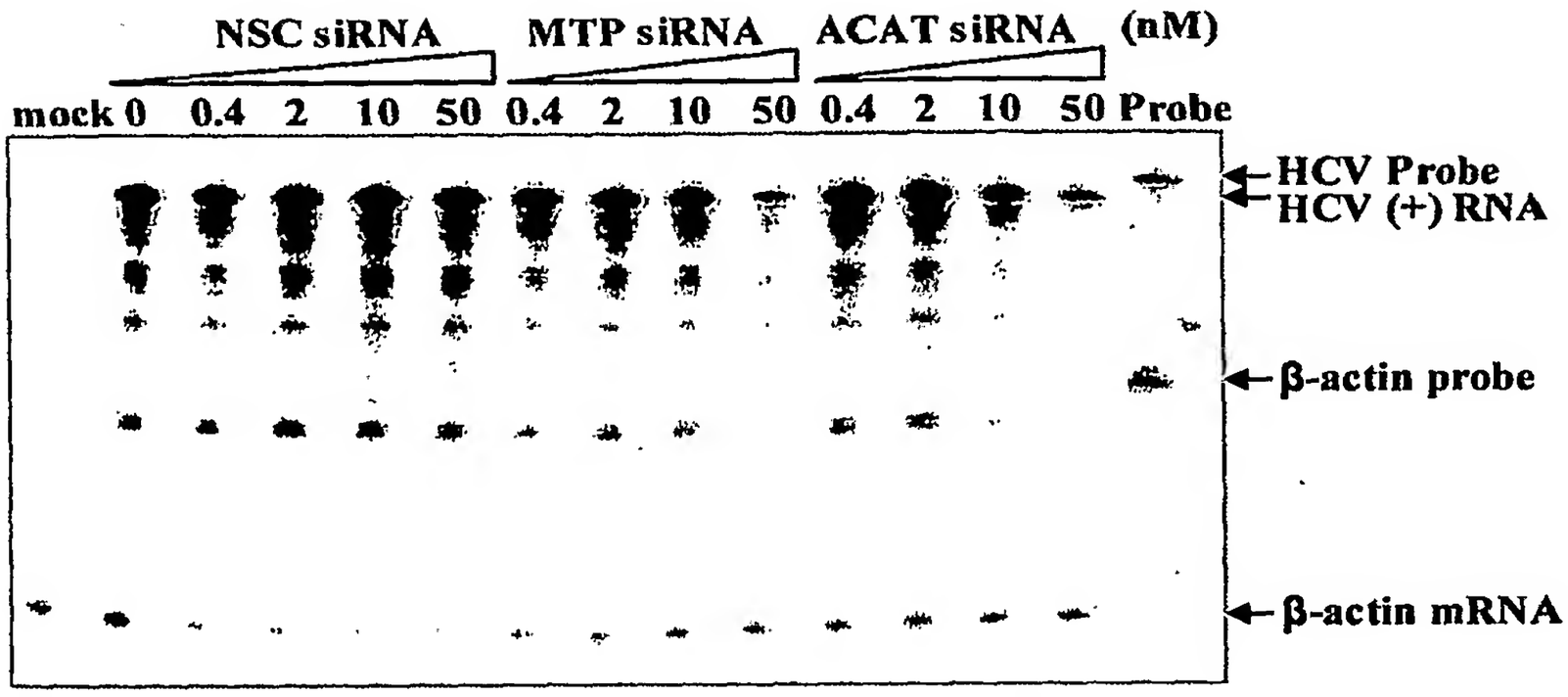


FIGURE 12A

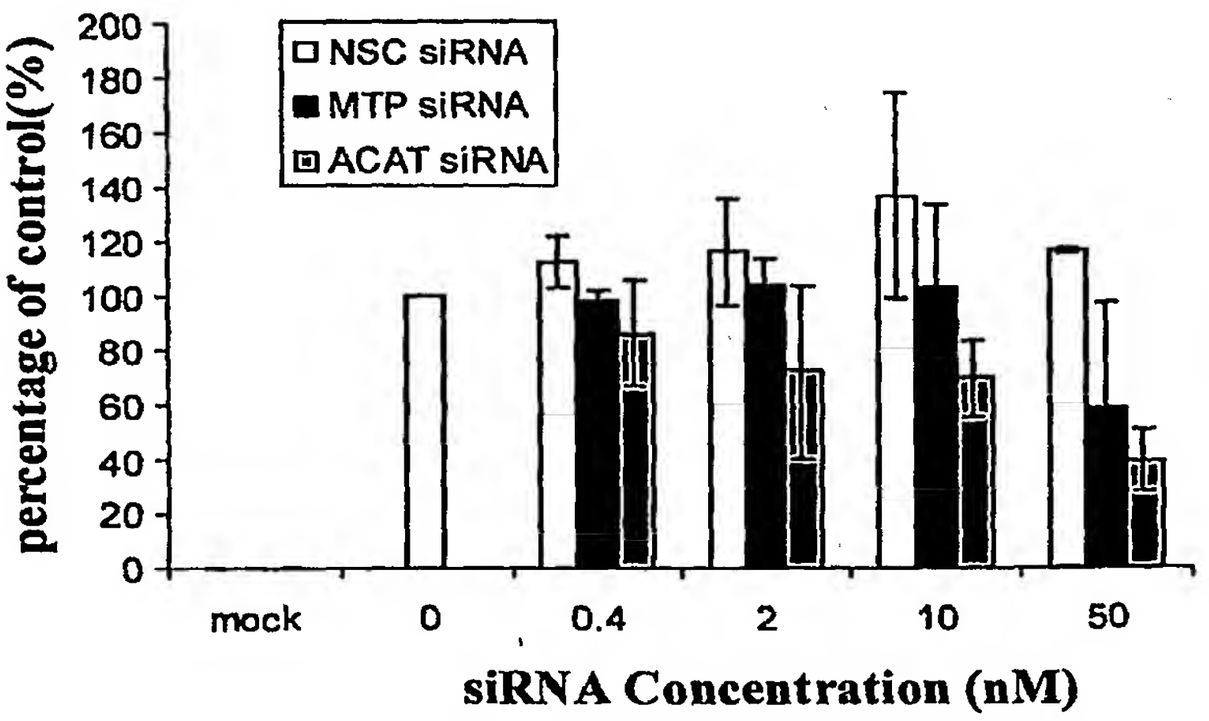


FIGURE 12B

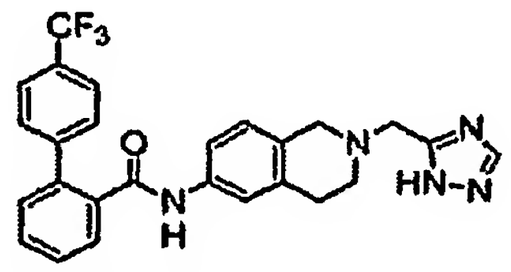
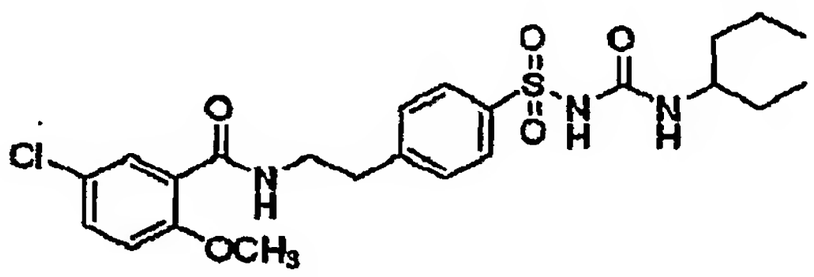
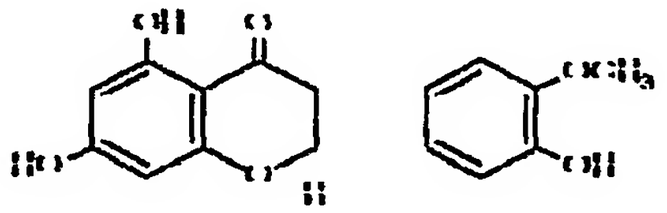
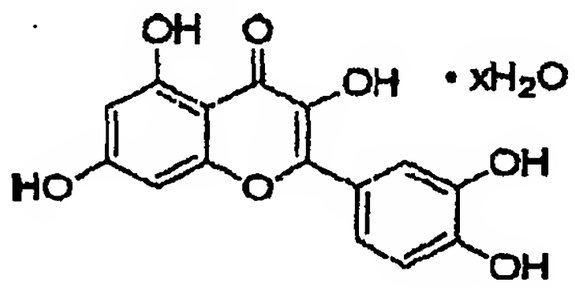
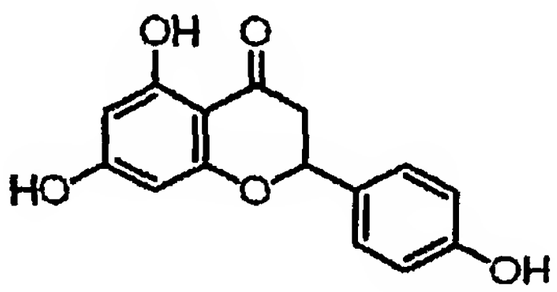
CP-346086	
Glybenclamide	
Hesperetin	
Quercetin	
Naringenin	

FIGURE 13

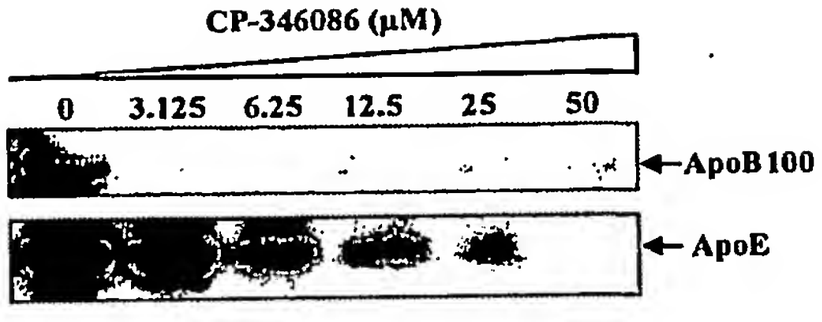


FIGURE 14A

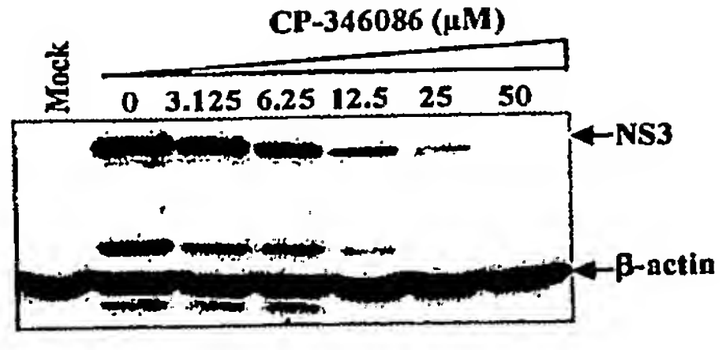


FIGURE 14B

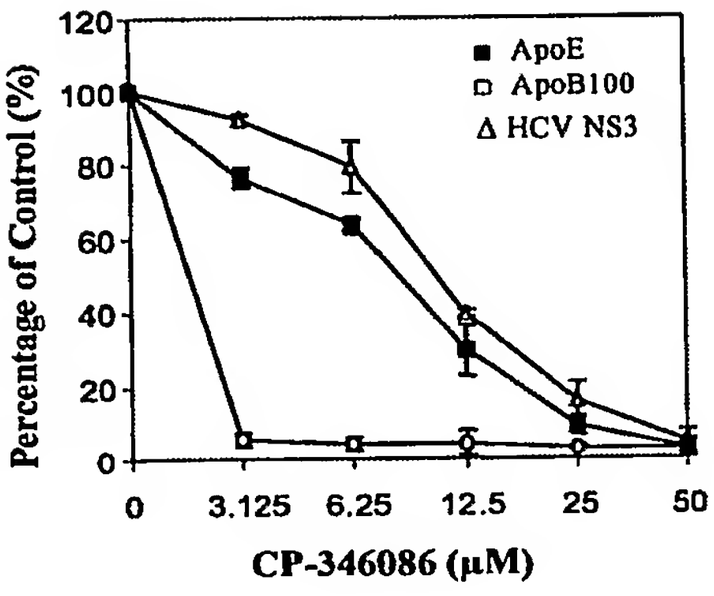


FIGURE 14C

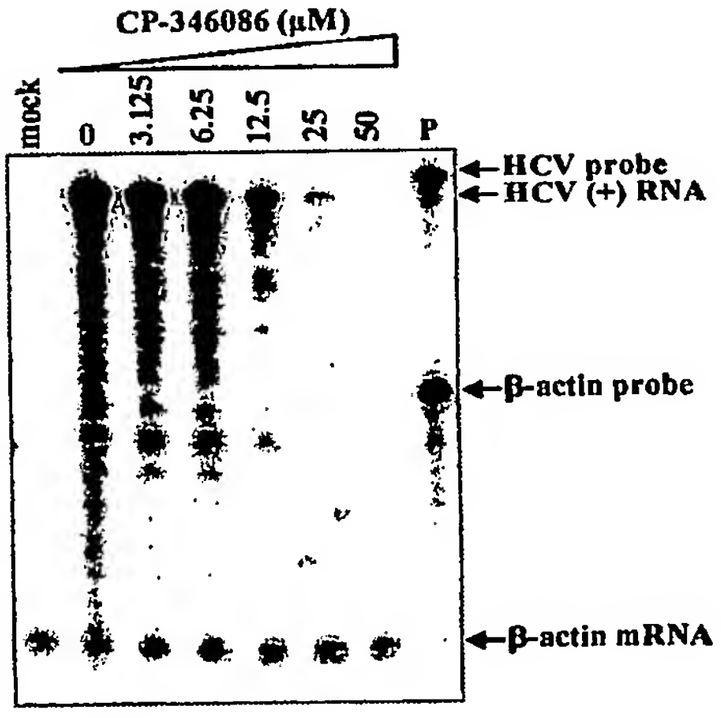


FIGURE 14D

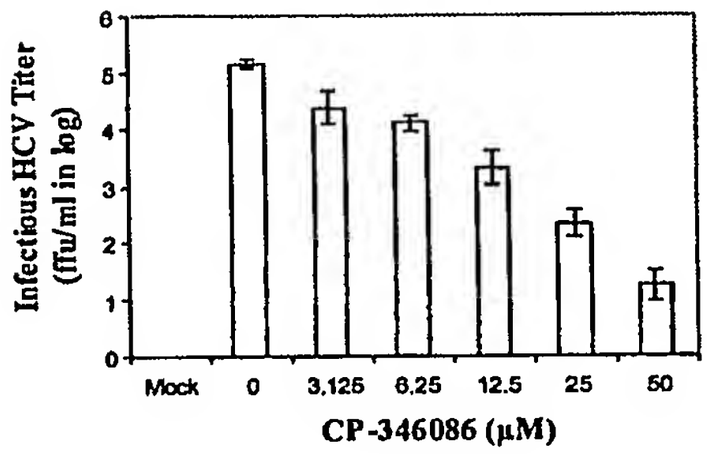


FIGURE 14E

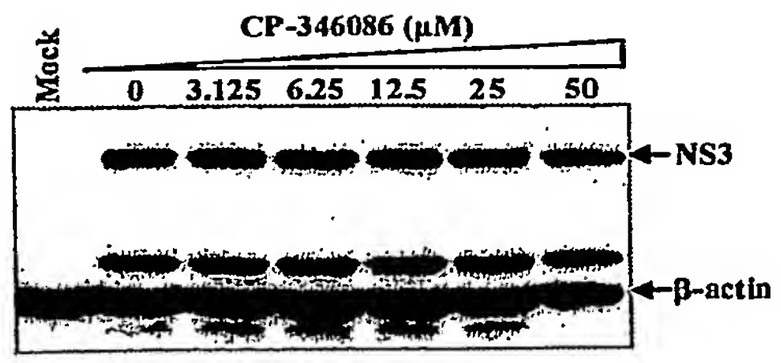


FIGURE 14E

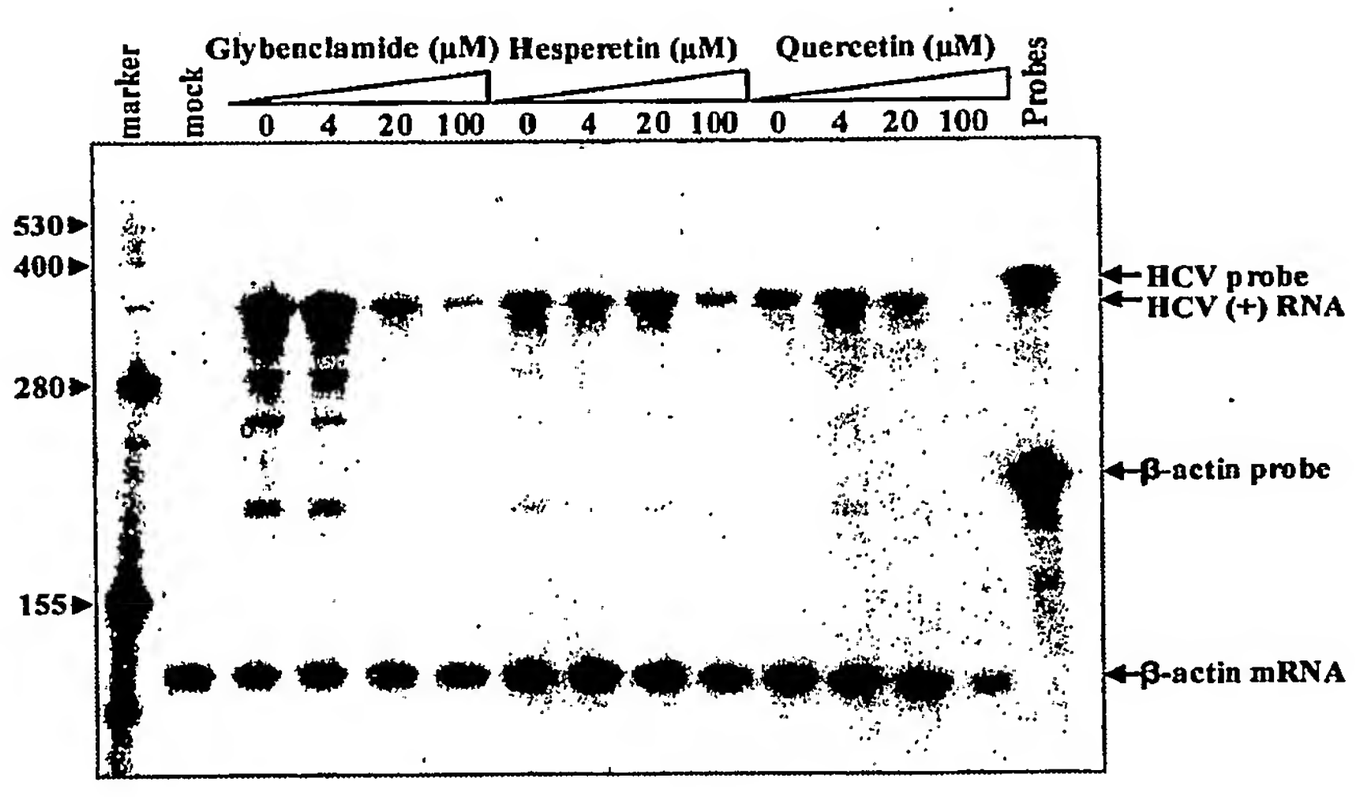


FIGURE 15

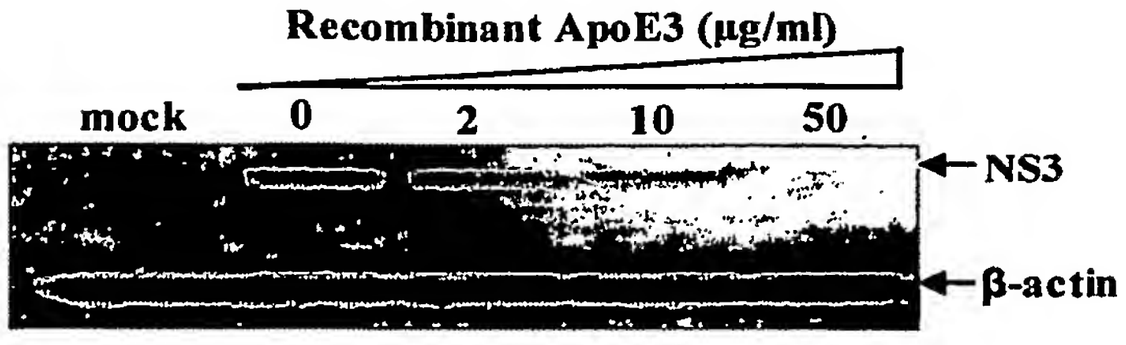


FIGURE 16A

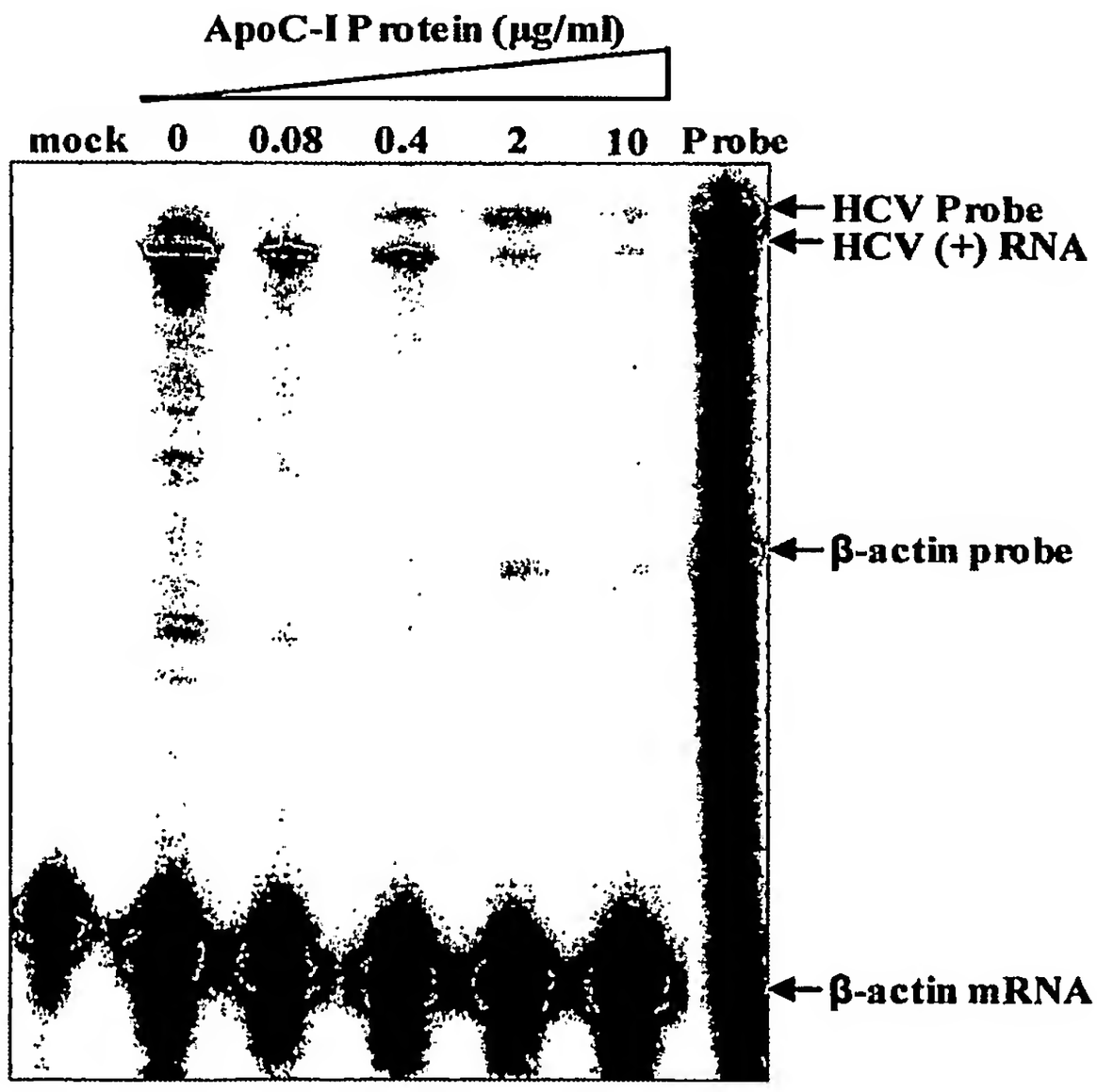


FIGURE 16B

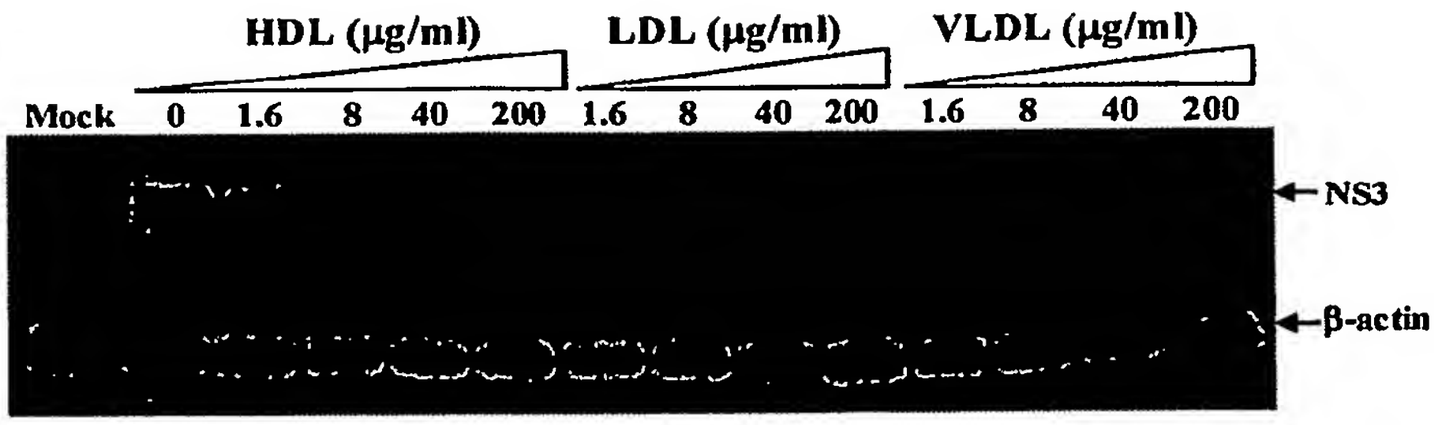


FIGURE 17A

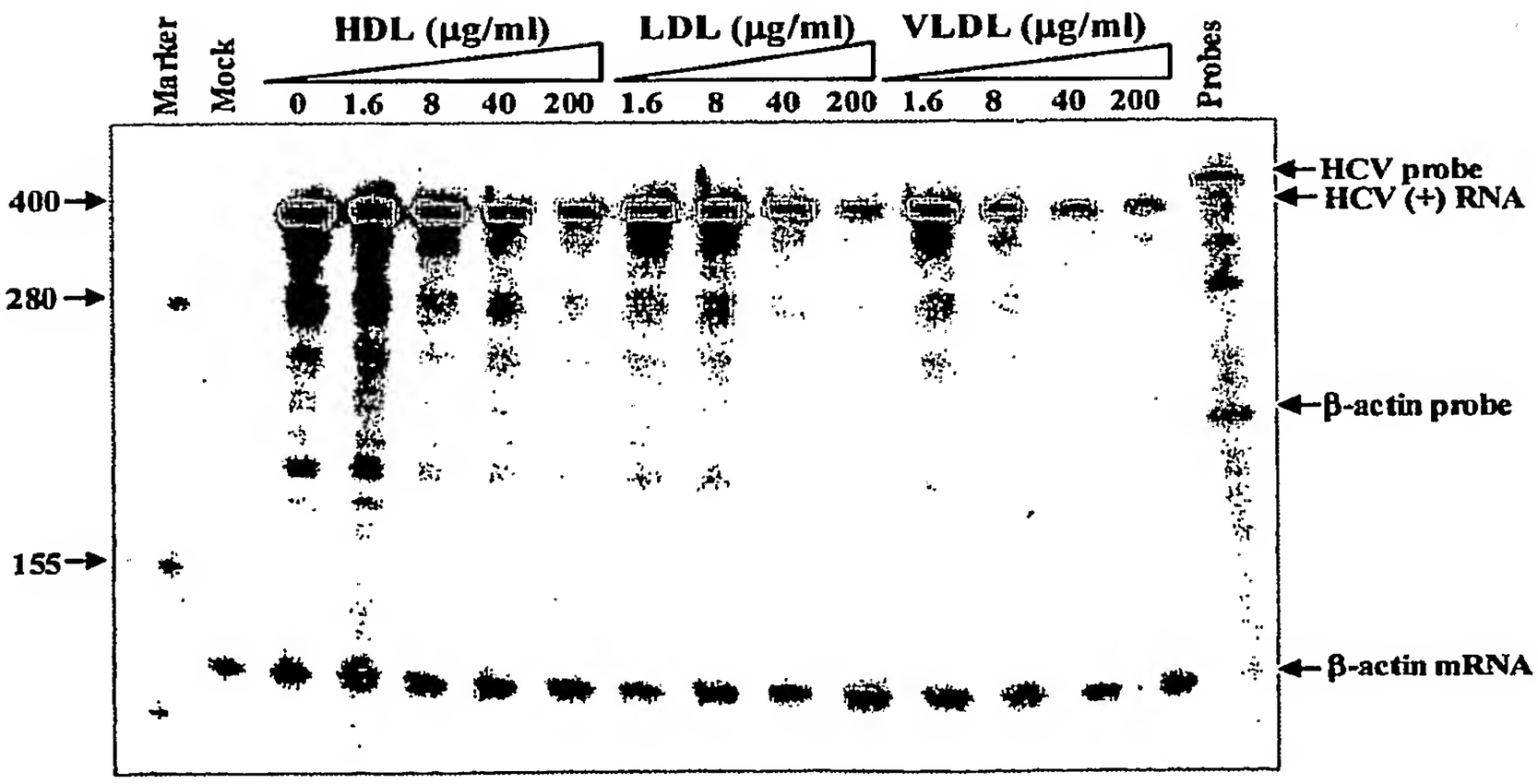


FIGURE 17B

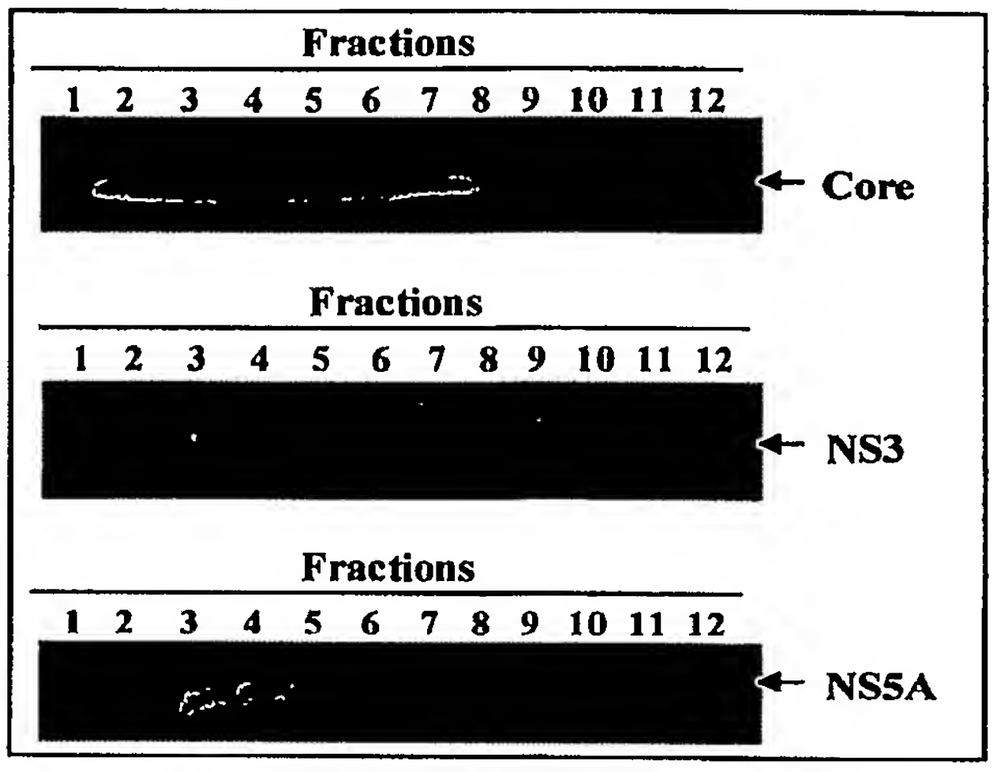


FIGURE 18

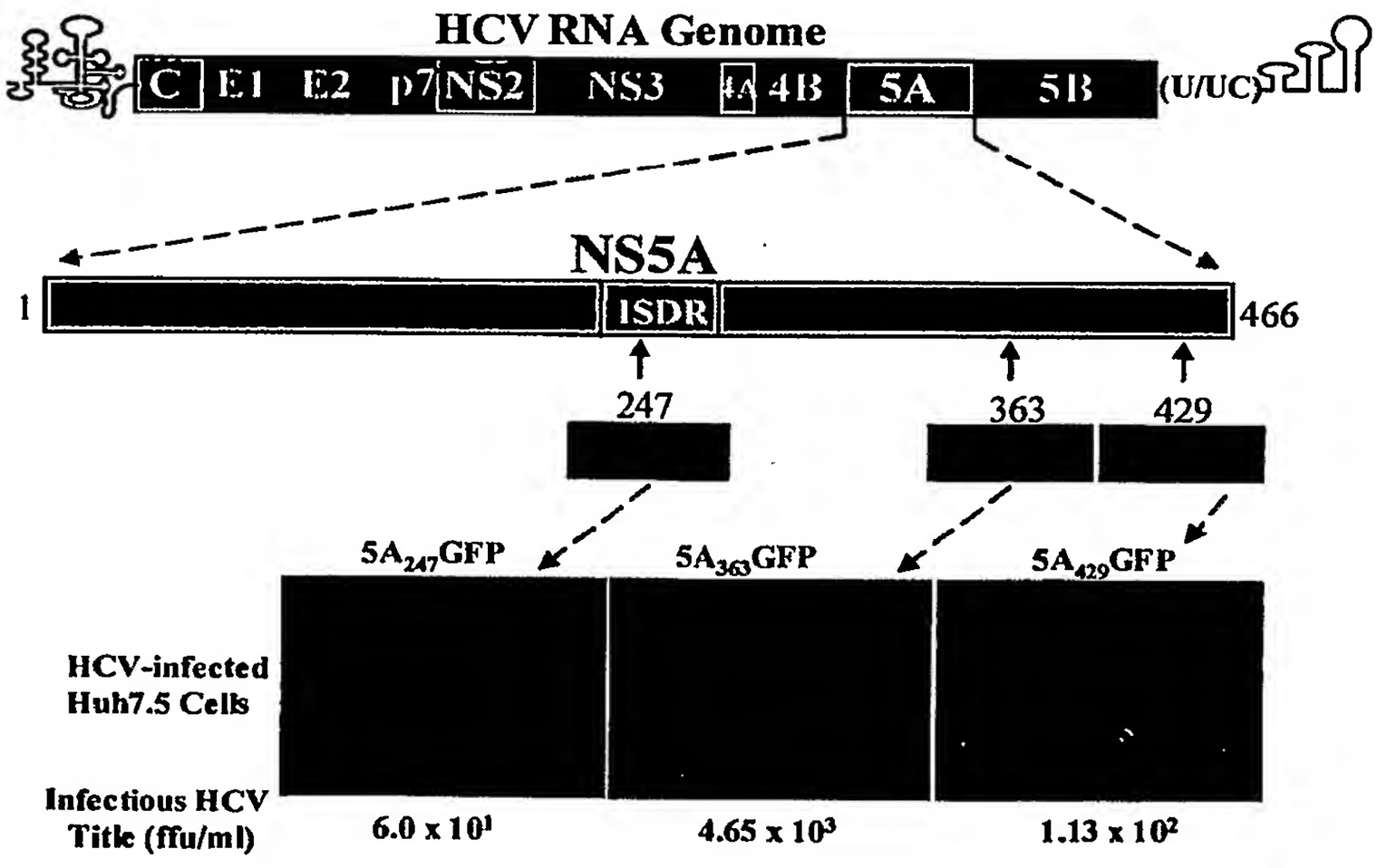


FIGURE 19A

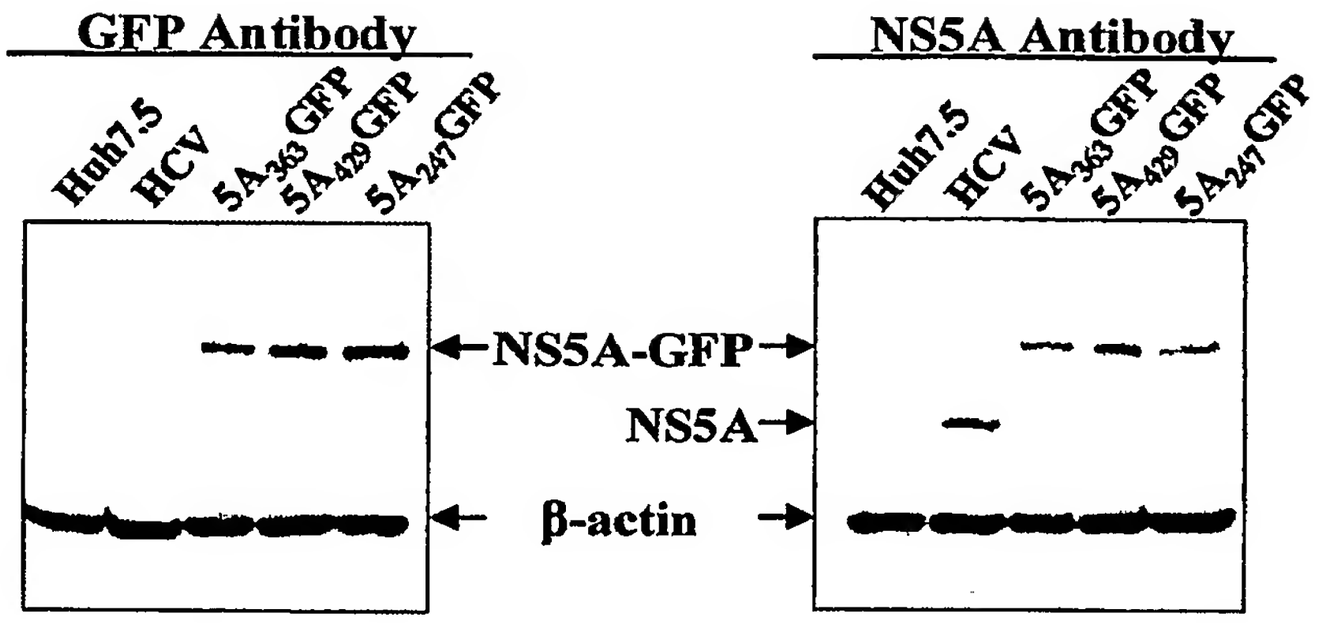


FIGURE 19B

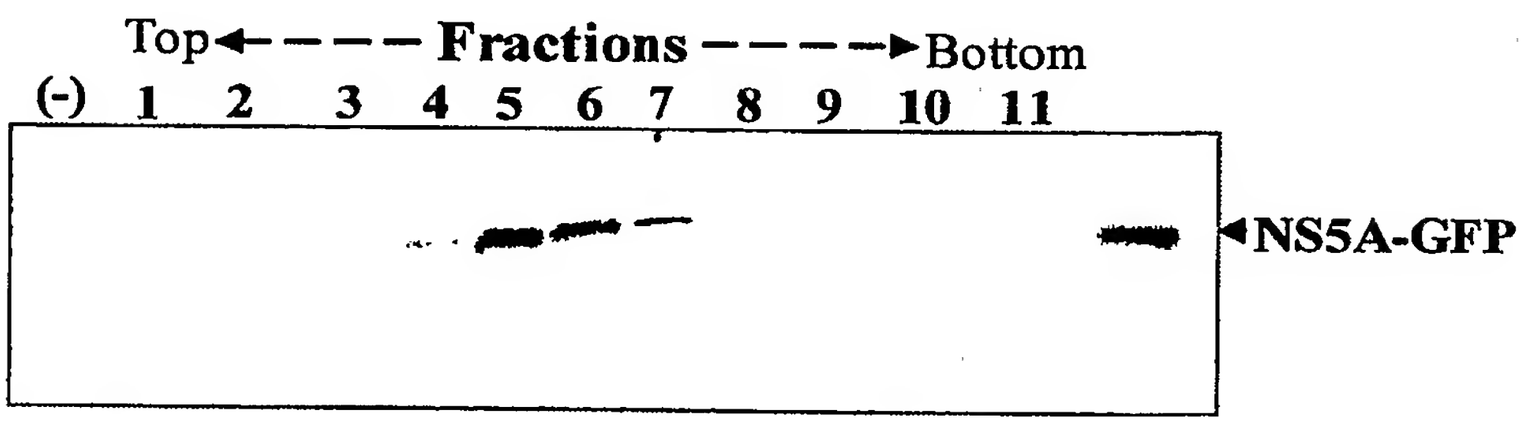


FIGURE 19C

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PCT/US2007/017970

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(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/822,354 14 August 2006 (14.08.2006) US

(71) Applicant and
(72) Inventor: **LUO, Guangxiang** [—/US]; 2456 Olde Bridge Lane, Lexington, KY 40513 (US).

(74) Agent: **TANKHA, Ashok**; Of Counsel, Lipton, Weinberger & Husick, 36 Greenleigh Drive, Sewell, NJ 08080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
23 April 2009

WO 2008/021353 A3

(54) Title: COMPOSITION AND METHOD FOR CONTROLLING HEPATITIS C VIRUS INFECTION

(57) Abstract: Disclosed herein are methods and compositions for the treatment and prevention of Hepatitis C Virus (HCV) infection and methods of screening for antiviral agents against HCV infection and/or production. A method of using compositions of certain apolipoprotein-specific monoclonal or polyclonal antibodies to inhibit HCV infectivity is disclosed. Further, methods of using small interfering RNAs (siRNAs) specific to apolipoproteins for treating and/or preventing HCV infection are disclosed. Also disclosed are methods of using siRNAs specific and/or small molecule inhibitors to certain lipoprotein biosynthetic genes and of using recombinant apolipoprotein E and/or their forms of lipoproteins to treat and/or prevent HCV infections. Screening methods for anti-HCV agents include assessing the effect of a candidate agent on apolipoprotein E and/or apolipoprotein C-I gene expression, assembly, and/or secretion and assessing the effect of a candidate agent on the blockage of the interaction and/or incorporation of HCV nonstructural proteins and/or their fusion forms with reporter proteins into HCV virions.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/17970

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **A61K 39/395**(2006.01);**C07K 16/00**(2006.01)

USPC: 424/130.1,141.1;530/387.1,388.1,388.25,389.1,389.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1, 141.1; 530/387.1, 388.1, 388.25, 389.1, 389.3,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PUBMED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	KRUL et al., "Roles of apolipoproteins B and E in the cellular binding of very low density lipoproteins," Journal of Clinical Investigation, Vol 75, pages 361-369 (February 1985).	1 ---------- 2
X --- A	MAURICE et al., "A potential complication in the use of monoclonal antibodies: inhibition of apoB-mediated receptor binding by an anti-apoE antibody," Journal of Lipid Research, Vol 30, pages 587-96 (1989)	1 ---------- 2
X --- A	RAFFAI et al., "Molecular characterization of two monoclonal antibodies specific for teh LDL receptor-binding site of human apolipoprotein E," Journal of Lipid Research, Vol 36, pages 1905-1918 (1995).	1 ---------- 2
A	ITZHAKI et al., "Apolipoprotein E and hepatitis C virus," Hepatology, Vol 38 No. 4, page 1060 (October 2003).	1,2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2008 (25.01.2008)	28 APR 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Zachariah Lucas Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/17970

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,T	CHANG et al., "Human Apolipoprotein E Is Required for Infectivity and Production of Hepatitis C Virus in Cell Culture," Journal of Virology, Vol. 81, No. 24, p. 13783-13793 (December 2007)	1,2
A	US 2005/0245502 (KELLER et al.) 03 November 2005 (03.11.1995), page 7 paragraph [0069].	1,2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/17970

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: Please See Continuation Sheet

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/17970

BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1 and 2, drawn to anti-apolipoprotein E antibodies, and a first method of use.

Group II, claim(s) 1 and 2, drawn to anti-apolipoprotein C-I antibodies, and a first method of use.

Group III, claim(s) 3-5, 11, and 12 drawn to apolipoportein directed siRNA, and first method of use.

Group IV, claim(s) 6-7, 11, and 12, drawn to siRNA directed to a lipoprotein biosynthetic gene.

Group V, claim(s) 8-12, drawn to a small molecule inhibitor of lipoprotein biosynthesis.

Group VI, claim(s) 13 and 14, drawn to a method for the identification of an anti-HCV agent by measuring the effect of the compound on apolipoprotein E expression.

Group VII, claim(s) 15, drawn to a method for the identification of an anti-HCV agent by measuring the effect of the agent on an HCV non-structural protein.This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

For Group IV, above, the three species are identified in claim 6, which identifies targets for the claimed siRNA- the species (a) acyl coenzyme A:cholesterol acyltransferase, (b) Cholesteryl Ester Transfer Protein, and (c) microsomal triglyceride transfer protein.

For Group V, the six species are identified in claim 9, which identifies targets for the small molecule inhibitor as species (a) acyl coenzyme A:cholesterol acyltransferase, (b) Cholesteryl Ester Transfer Protein, and (c) microsomal triglyceride transfer protein, (d) the gene responsible for apoE synthesis, (e) the gene responsible for apoE assembly, and (f) the gene responsible for apoE secretion.

The claims are deemed to correspond to the species listed above in the following manner:

The species of Group IV are found in claim 6. The species of Group V are found in claim 9.
The following claim(s) are generic: For Group IV, claim 11 is generic. For Group V, claims 8 and 11 are generic.

The inventions listed as Groups I-VII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the common technical feature of the claims is that they are all related to anti-HCV agents. The claims identify antibodies against apolipoprotein E as such an agent, and the

specification teaches on page 15 that the mab A1.4 is such an agent. Compositions comprising this agent are found in the teachings of MAURICE et al., Journal of Lipid Research, Vol 30, pages 587-96 (1989). Thus, the claimed inventions do not share a common special technical feature.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the various species each relate to the use of inhibitors of lipoprotein biosynthesis for the treatment of HCV. One such inhibitor identified by the application is quercetin, which compound is taught as useful for the treatment of HCV by KELLER et al. U.S. 2005/0245502, page 7 paragraph [0069]. Thus, the species do not share a common special technical feature.

Continuation of Box III Item 4:
1 and 2 (wherein the compostion comprises an antibody that recognized apolipoprotein E).